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# **THE ASSESSMENT OF VITAMIN D, ANTIMICROBIAL PEPTIDES AND PROCALCITONIN IN BRONCHIECTASIS**

A thesis  
submitted in partial fulfilment  
of the requirements for the degree  
of  
**Masters of Science in Biological Sciences**  
at  
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by  
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The University of Waikato  
2014



THE UNIVERSITY OF  
**WAIKATO**  
*Te Whare Wānanga o Waikato*

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# Abstract

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My research investigated the distribution of antimicrobial peptides (cathelicidin and  $\beta$ -defensin 2), vitamin D and the inflammatory marker procalcitonin, between systemic and localised respiratory levels in stable bronchiectasis patients.

Bronchiectasis is a localised chronic lung disease that is characterised by acute reoccurring lower tract respiratory infections. Non-cystic fibrosis bronchiectasis patient serum and sputum samples were collected as part of an ongoing clinical trial. This trial is investigating the usefulness of Tiotropium for the treatment of bronchiectasis.

Antimicrobial peptides are a fundamental component of the innate immune system. The innate immune system is vital in providing the first line of defence against pathogens. At epithelia sites this is more so, as cells are often in direct contact with the environment. Both cathelicidin and  $\beta$ -defensin 2 expression has been found to be upregulated by the active vitamin D metabolite, 1,25-dihydroxyvitamin D. This research also aimed to determine if vitamin D deficiency is prevalent in bronchiectasis, and how these levels correspond with cathelicidin and hBD-2. Lastly, whether procalcitonin, a known inflammatory marker of bacterial infections is a suitable biomarker for bronchiectasis. Other inflammatory markers have been shown to be consistently elevated in stable bronchiectasis patients.

The use of commercial ELISA kits demonstrated the notion of systemic levels not being representative of localised levels. Both of the antimicrobial peptides, cathelicidin and  $\beta$ -defensin 2, show elevated sputum levels consistent with bronchiectasis being a localised condition. Contrastingly, vitamin D metabolites are found to be lower in the airways. This is predicted to be due to the majority of vitamin D metabolites being protein bound in circulation, thereby affecting their tissue distribution. Stable bronchiectasis patients do not have persistently elevated

serum procalcitonin levels but procalcitonin levels are significantly higher in the sputum.

Our findings report that suboptimal vitamin D levels, but not vitamin D deficiency, were prevalent in stable bronchiectasis patients. No correlations were found between 25-hydroxyvitamin D and antimicrobial peptide concentrations, consistent with other studies findings. However, localised levels of the active form, 1,25-dihydroxyvitamin D, were correlated with localised cathelicidin (CAMP) levels but not  $\beta$ -defensin 2.

Future work should focus on the hormonal form, 1,25-dihydroxyvitamin D, if an *in vivo* association with antimicrobial peptides is to be found. A number of factors can influence vitamin D bioavailability, including, known single nucleotide polymorphisms in the vitamin D receptor and vitamin D binding protein.

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# Abbreviations

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<b>1,25(OH)<sub>2</sub>D</b>	1,25-dihydroxyvitamin D
<b>1,25(OH)<sub>2</sub>D<sub>3</sub></b>	1,25-dihydroxyvitamin D <sub>3</sub>
<b>25(OH)D</b>	25-hydroxyvitamin D
<b>25(OH)D<sub>3</sub></b>	25-hydroxyvitamin D <sub>3</sub>
<b>AAT</b>	α-1-antitrypsin
<b>ABPA</b>	Allergic bronchopulmonary aspergillosis
<b>AMP</b>	Antimicrobial peptide
<b>BAL</b>	Broncho alveolar lavage
<b>bp</b>	Base pairs
<b>Calu-3</b>	Lung adenocarcinoma cells
<b>CAMP</b>	Cathelicidin antimicrobial peptide gene
<b>CF</b>	Cystic fibrosis
<b>CoA</b>	Certificate of analysis
<b>COPD</b>	Chronic obstructive pulmonary disorder
<b>CRP</b>	C-reactive protein
<b>CT</b>	Computed tomography scan
<b>CVID</b>	Common variable immune deficiency
<b>CYP24A</b>	24-hydroxylase enzyme
<b>CYP27A1/CYP2R1</b>	25-hydroxylase enzyme
<b>CYP27B1</b>	1-α-hydroxylase enzyme
<b>DBP</b>	Vitamin D binding protein
<b>ddH<sub>2</sub>O</b>	De-ionized water
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>ESR</b>	Erythrocyte sedimentation rate
<b>FEV<sub>1</sub></b>	Forced expiratory volume in one second
<b>hBD-2</b>	Human beta-defensin 2
<b>hCAP18</b>	Human cathelicidin antimicrobial peptide
<b>HRCT</b>	High-resolution computed tomography
<b>HRP</b>	Horseradish peroxidase

<b>HrQoL</b>	Health-related quality of life questionnaire
<b>HVC</b>	High value control
<b>IBD</b>	Inflammatory bowel disease
<b>IL-1<math>\beta</math></b>	Interleukin-1B
<b>IU</b>	International units
<b>LCQ</b>	Leicester cough questionnaire
<b>LL-37</b>	Mature cathelicidin peptide
<b>LPS</b>	Lipopolysaccharide
<b>LTRI</b>	Lower tract respiratory infection
<b>mRNA</b>	Messenger ribonucleic acid
<b>MVC</b>	Middle value control
<b>NTM</b>	Nontuberculosis mycobacteria
<b>OD</b>	Optical density
<b>PAMP</b>	Pathogen-associated molecular pattern
<b>PBS</b>	Phosphate buffered saline
<b>PCD</b>	Primary cilia dyskinesia
<b>PCT</b>	Procalcitonin
<b>PRP</b>	Pathogen recognition receptors
<b>PTH</b>	Parathyroid hormone
<b>RXR</b>	Retinoid X receptor
<b>SCC25</b>	Head and neck squamous carcinoma cells
<b>SGRQ</b>	St. Georges respiratory questionnaire
<b>TLR</b>	Toll-like receptor
<b>TMB</b>	Tetramethylbenzidine
<b>U937</b>	Myelomonocytic cells
<b>UVB</b>	Ultraviolet B
<b>VDD</b>	Vitamin D deficiency
<b>VDR</b>	Vitamin D receptor
<b>VDRE</b>	Vitamin D response element

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# Chapter One: Introduction and Literature Review

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## 1.1 Bronchiectasis

Laennec first described the existence of bronchiectasis in 1819 [1]. Bronchiectasis has since been defined as a chronic lung disease that can be characterised by the persistent dilatation of the bronchi [2]. Common symptoms of bronchiectasis include a persistent cough, purulent mucus production, dyspnoea (breathlessness), haemoptysis (coughing up of blood), wheezing, crackles, chest pain, weight loss, rhinosinusitis, finger clubbing and general fatigue [3, 4].

Patients living with bronchiectasis experience reoccurring lower tract respiratory infections (LTRI's) due to their increased susceptibility to infection and microbial colonization [5, 6]. These exacerbations often require frequent hospitalization and cause progressive loss of lung function [7]. Sufferers live with a high level of morbidity and have a high risk of premature mortality [6, 8-10]. A New Zealand study reported a mortality rate in bronchiectasis patients of 21% (32/152) over twelve months [10]. A Finnish study of 842 individuals (diagnosed in 1982-1986) revealed a mortality rate of 28% (longest follow up period was 12.9 years) [6]. Bronchiectasis is a significant burden on those it affects, greatly limiting their quality of life [6].

### 1.1.1 Exacerbations

There is no standardized definition of an exacerbation available for bronchiectasis [11, 12]. Bronchiectasis exacerbations are commonly compared to chronic obstructive pulmonary disorder (COPD), usually using increased breathlessness and purulent sputum production as indicators [11]. Typically an exacerbation is any period of deterioration seen in respiratory condition [13]. Exacerbations often present as a worsening of the broad symptoms, for example, increased sputum



production, deteriorated cough or change in cough character [14]. The definition of exacerbations for adults is different from that required for children [15]. Severe exacerbations will require hospitalization and the use of intravenous or oral antibiotics [16]. The rate of hospitalizations due to exacerbations is increasing. The average US hospitalisation rate for bronchiectasis from the years 1993 to 2006 is 16.5/100,000 and has increased approximately 3% every year [17].

Research indicates an exacerbation rate of 1.5-6.5 per year for bronchiectasis patients [18, 19]. A contemporary study of 152 patients showed there was a total of 307 exacerbations, of which at least 46% were readmitted within another exacerbation in the year [10]. Another 2009 study identified 115 exacerbations in 30 children during their study period. This gave a rate of 1.6 exacerbations per year, of which 35% of exacerbations required hospitalization and treatment with intravenous antibiotics [16]. Currently there is an absence of specific bronchiectasis measures for exacerbations. This presents a need for serum or sputum biomarkers to be identified, to aid in the monitoring of exacerbations [11].

### **1.1.2 Diagnosis**

Bronchiectasis is often difficult to diagnose and therefore is commonly misdiagnosed [4, 20]. Bronchiectasis most often gets mistaken for other common respiratory conditions, for example, asthma or COPD. A delay usually exists between the onset of bronchiectasis and a confirmed diagnosis. One bronchiectasis phenotyping study has shown a delay in diagnosis of 17 years. The mean age of diagnosis was 54 years, yet the mean age of onset of symptoms was 37 years [21]. Symptoms often first present themselves in childhood; however a diagnosis is often not obtained until adulthood.

The current 'gold standard' for diagnosis is the use of high resolution computed tomography (HRCT) scans [3, 22]. This is a non-invasive procedure [23] used to confirm the dilation and thickening of bronchial walls, as seen in Figure 1 [3]. HRCT confirms bronchiectasis if the bronchi are internally dilated by more than 1.5 times the pulmonary artery size [23, 24]. The HRCT scan will identify a lack airway tapering [23], thickened bronchial walls, airway constrictions and cysts at

the bronchial end points [3]. These abnormalities can similarly indicate the occurrence of bronchiectasis. Even now with the availability of HRCT scans, the overall diagnosis of bronchiectasis is still poor in both developed and developing countries [14].



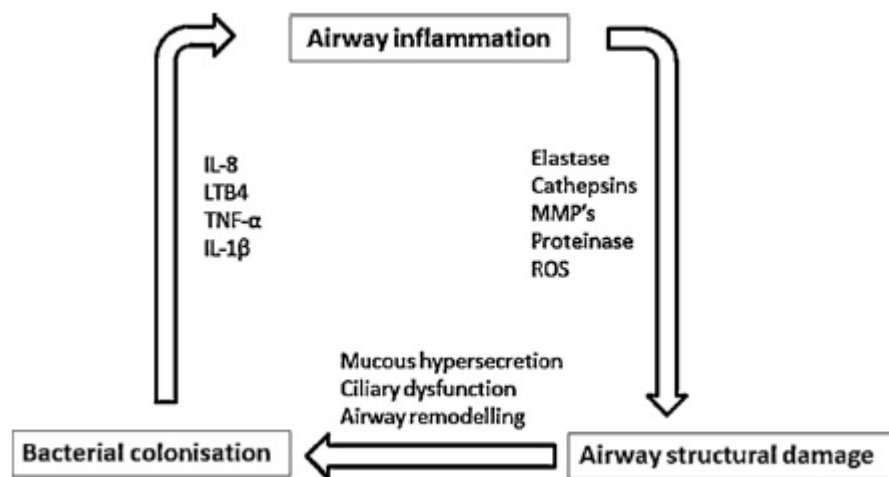
**Figure 1** High-resolution computed tomographic (HRCT) image of bronchiectasis lungs showing dilated and thickened airways, as published by Barker, 2002 [3]

### **1.1.3 Pathophysiology**

Bronchiectasis arises due to a pattern of continual cycles of infection, followed by periods of persistent inflammation in the airways. This ‘vicious cycle’, a term coined by Coles in 1986, is a chain of events that leads to progressive damage of connective tissue and cilia within the bronchi. This destruction leads to the general obstruction of smaller airways and affects the mucociliary-clearance function. Thus the lungs lose their vital natural protective mechanisms, placing them at risk of pathogen growth and colonization. This cycle occurs after an initial

insult which can either be environmental, congenital or as a result of immune defects [5].

Coles 'vicious cycle' is a host-facilitated phenomenon and works on the principle that inflammation has the potential to be both, beneficial and detrimental. Normally inflammation is a brief controlled response upon exposure to foreign material that is used to provide a line of protection. But if elimination of the pathogen is not successful, then this can lead to chronic inflammation. Chronic inflammation limits the repair response of the lungs, thereby allowing damage to occur in healthy tissue. This cycle results in the permanent dilatation of the bronchi [5].



**Figure 2** The 'vicious cycle' hypothesis of bronchiectasis, as published by Chalmers et al., 2013 [5, 25]

Bronchiectasis is regarded as a neutrophil driven disease [25, 26]. During each exacerbation, particularly in severe ones, neutrophils migrate to the respiratory tract. This recruitment of neutrophils is stimulated by chemoattractants, for example, interleukin 8, tumour necrosis factor- $\alpha$  or interleukin 1B (IL-1 $\beta$ ). The neutrophils then act to increase the levels of proteolytic enzymes [25]. Studies show that both cytokines and proteolytic enzymes (eg. MMP-8 or elastase) have been found to be upregulated in stable bronchiectasis patients [25, 27-29]. It is the process of infection, neutrophilic inflammation and proteolytic enzymes that work

in a cycle to damage the airways, leading to the development of bronchiectasis [13]. This forms the 'vicious cycle' hypothesis of bronchiectasis (Figure 2).

#### **1.1.4 Prevalence/Incidence**

The prevalence of bronchiectasis appeared to be decreasing during the twentieth century, where the aforementioned became regarded as an 'orphan' disease [6, 13]. Recently a resurgence of bronchiectasis has been noted within certain communities [30]. There is very limited data available worldwide that has measured the prevalence and incidence of bronchiectasis. Understandably this makes comparing data from different prevalence studies difficult. The underestimation of bronchiectasis is a major issue, as most individuals are outpatients and often are misdiagnosed with COPD or asthma [4, 13]. The notion stands that bronchiectasis is a neglected and poorly understood condition, placing limitations on its management [14].

A recent two year study of bronchiectasis in New Zealand children provides the most recent national prevalence rates. The investigation revealed an estimated national incidence rate of 3.7/100,000 per year, in children less than 15 years of age. This figure, due to study design, should be regarded as the minimum incidence figure. The only other comparable data available is the national incidence rate from a contemporary study of under 15 year olds in Finland. Their estimated incidence rate is 0.5/100,000 per year [31]. The New Zealand's national incidence rate in under 15 year olds is seven times the national incidence rate in Finland. These two studies similarly highlight the concept that bronchiectasis most often develops during early childhood; but a recognised significant delay in diagnosis.

Interestingly, the New Zealand study identified ethnic and regional variances in the incidence of bronchiectasis. An incidence of 17.8/100,000 in Pacific Island children and 4.8/100,000 in Maori children was reported. These incidences are twelve times and three times higher, respectively, than for NZ European children [9]. These ethnic findings were also supported by older New Zealand data reporting a prevalence of 1/6,000 children [32]. Similar findings have been

reported in other indigenous populations. A rate of 16/1,000 is reported in Southwest Alaskan Native Children from Yukon Kuskokwim Delta region [33]. Another study shows an incidence of 15/1,000 in central Australian Aboriginal children [34]. In both of these populations respiratory disease is the largest preventable cause of death in infants [33, 34]. Limited data is available for the prevalence of bronchiectasis in New Zealand adults [10].

Multiple studies have found a predisposition for the development of bronchiectasis in women and at the extremes of age [9, 23, 35]. Those greatest at risk are therefore elderly women [36]. In regards to the extremes of age, a US study has shown an increase from 4.2/100,000 in 18-34 year olds to 271.8/100,000 in over 75 year olds [36]. The higher frequency of bronchiectasis in females is supported by the recent New Zealand incidence study, where 37 out of 65 participants were female [9]. Pasteur's bronchiectasis pathogenesis study showed a larger majority of female (n=94) than male counterparts (n=56). Additionally in this study, females also had a higher mean age [23]. In Shoemark's bronchiectasis aetiology research, a total of 65% of the participants were female [35]. There is an indication for a predisposition in women for the development of bronchiectasis; however the disease has been shown to progress equally in both sexes.

Other global prevalence rates include the Hong Kong government estimate of 16.4/100,000 in 1990 [13]. In the US 110,000 adults were determined to have bronchiectasis in a 2005 study [36]. Newer US data indicates a rate of 52/100,000 at an additional cost of 1.1 billion dollars per annum [17]. This study also showed that the prevalence of bronchiectasis increasing by 8.7% per year between 2007 and 2008 in the US [17]. Clearly, bronchiectasis prevalence is on the rise and should now be regarded as a 'common' disease worldwide [30].

### **1.1.5 Aetiologies**

A substantial number of causes can be linked to the onset of bronchiectasis. Finding a causative factor is vital in the long-term management of the condition, as it has the potential to influence therapeutic options. There are a number of

papers investigating the pathogenesis of bronchiectasis [9, 21, 23, 35]. Many of these studies, shown in Table 1, involved specialist respiratory clinics [23, 35]. Consequently, these bronchiectasis cases are likely to be more complex than other studies, composed mainly of individuals struggling to control symptoms. These studies may not accurately represent stable bronchiectasis aetiologies [21, 35]. The recent phenotyping study by Anwar *et al.*, 2013 has used a more generalised sample population, thereby reducing this bias [21].

The majority of cases (50-80%) of bronchiectasis are deemed to be idiopathic; no known causative factor [23]. Most idiopathic instances are probably due to unknown immunological mechanisms. There is beginning to be a move away from focusing on extrinsic factors (eg. post-infection) to more intrinsic factors (eg. congenital, systemic or immunological) [23].

Post-infection is the next most prevalent contributing factor in providing the initial insult for bronchiectasis. This includes various childhood respiratory conditions, for example, pneumonia, tuberculosis, whooping cough, adenovirus, complicated measles and pertussis [3, 4]. The contribution of these conditions is probably not as relevant today with immunizations and use of antibiotics, so will expect to see a fall in these causalities [3, 23]. Post-infection is still common in indigenous populations and non-affluent countries [14]. Socioeconomic factors may also contribute to these health inequalities. These include low immunisation rates, overcrowded housing and poor access to healthcare [9, 33].

Congenital causes only make up a small proportion but often therapy can be taken in these instances. Cystic fibrosis (CF) is a well-known autosomal recessive disorder; most predominant in white populations with a rate of 1 in every 2500 births [13]. Bronchiectasis is a significant factor in CF mortality [37]. This research is only focusing on non-cystic fibrosis bronchiectasis. Other congenital causes include  $\alpha$ -1-antitrypsin (AAT) deficiency and allergic bronchopulmonary aspergillosis (ABPA) [13, 22]. ABPA is characterised as a hypersensitive response to the bacteria *Aspergillus* in the airways of asthmatics; can result in the development of bronchiectasis in the upper lobe of the lung [13].

**Table 1 Comparison of existing phenotyping studies in bronchiectasis**

<b>Aetiology</b>	<b>Pasteur et al., 2000 [23]</b>	<b>Shoemark et al., 2007 [35]</b>	<b>Twiss et al., 2012 [9]</b>	<b>Anwar et al., 2013 [21]</b>
Number of Patients	150	165	65 (<15 yo)	189
Idiopathic	53%	26%	54%	43%
Post-Infectious	29%	32%	22%	24%
Immune Defects	8%	7%	6%	1%
Cystic Fibrosis	3%	1%	n/a	<1%
Young's syndrome	3%	3%	n/a	<1%
Ciliary dysfunction/PCD	1.5%	10% (PCD)	n/a	1%
Aspiration/Gastro- oesophageal reflux	4%	1%	6%	1%
Pan bronchiolitis	<1%	2%	n/a	n/a
Congenital Defect	<1%	n/a	n/a	n/a
ABPA	7%	8%	n/a	4%
Rheumatoid Arthritis	3%	2%	n/a	5%
Ulcerative Colitis	<1%	3%	n/a	2%
Yellow Nail Syndrome	n/a	2%	n/a	n/a
Mycobacterium infection	n/a	2%	n/a	n/a
Post-Oncology	n/a	n/a	11%	n/a
COPD	n/a	n/a	n/a	12%
Pink's Disease	n/a	n/a	n/a	<1%
AAT deficiency	0%	n/a	n/a	1%
Asthma	n/a	n/a	n/a	3%

Congenital ciliary disorders can lead to the development of bronchiectasis, for example primary cilia dyskinesia (PCD) and Kartagener's syndrome. PCD is a rare (1 in 15000-35000) autosomal recessive disorder that results in abnormally functioning cilia [38]. Cilia are vital structures located on mucosal surfaces, especially the respiratory tract. Their normal function is to beat and propel mucus, this helps to maintain a sterile airway environment. PCD therefore limits the

removal of debris and microorganisms, potentially leading to the development of bronchiectasis [3, 22].

Immune deficiencies or systemic conditions are beginning to be more associated with bronchiectasis, particularly in affluent countries [14]. Immunodeficiency is most often due to deficiencies in immunoglobulins (eg. IgG or IgM subclass deficiencies), humoral factors (eg. antibody deficiency or defective antibody response) or common variable immune deficiency (CVID) [3, 23, 35].

Bronchiectasis is often the end path of various systemic and respiratory diseases [13]. Bronchiectasis is often diagnosed in patients with inflammatory bowel disease (IBD) including, Crohn's disease, and ulcerative colitis; all of which are manifestations of the bowel [3]. The occurrence of bronchiectasis is also commonly associated with rheumatoid arthritis (a collagen vascular disease) [3, 19, 21, 35]. Panbronchiolitis is an obstructive airway disease found predominantly in Japanese individuals that is determined by the presence of parenchymal nodules. Panbronchiolitis also known to contribute to the onset of bronchiectasis, particularly in Japanese populations [13].

Comorbidities are a common feature in bronchiectasis patients, especially asthma and COPD. In individuals with COPD it has been shown that up to 50% may also have bronchiectasis on CT scans [39]. In fact a combination of bronchiectasis and COPD represent a more severe phenotype [40]. Comorbidities are regarded as risk factors as they increase the chance of mortality [6]. Other aetiologies include aspiration or obstruction of foreign bodies. This can include gastric reflux, tumours, foreign bodies or enlarged lymph nodes [13].

#### **1.1.5.1 Pathogens**

Microbial organisms are commonly isolated from bronchiectasis individuals. King *et al.*, 2007 study assessed the sputum of 89 clinically stable bronchiectasis patients. Initially the two most abundant pathogens were *Haemophilus influenzae* (47%) and *Pseudomonas aeruginosa* (12%) [41]; similar figures have been reported in a pathogenesis study of 35% and 31% respectively [23]. Meanwhile



21% of those studied had no pathogens isolated. Results remained fairly similar in follow up assessments, indicating chronic colonization; except for an increase in antibiotic resistance (from 13% up to 30%) [41]. Other isolated pathogens from bronchiectasis patients include *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Nontuberculosis mycobacteria* (NTM) [22, 41].

Some studies have indicated an association between the severity of bronchiectasis and chronic colonization of *Pseudomonas aeruginosa* [23, 40, 41]. This pathogen is typically very difficult to eradicate. Studies show an observed increased exacerbation rate and a decreased lung function (%FEV<sub>1</sub>) in patients chronically colonized by *Pseudomonas aeruginosa* [23, 41]. Chronic colonization by *Pseudomonas aeruginosa* is a high risk factor for mortality [8]. Patients that had no pathogens isolated from their sputum tended to show the mildest form of disease, whilst those colonised by *Pseudomonas aeruginosa* the most severe [41]. Remarkably pathogens are not found in all individuals, even during acute exacerbations. Often pathogens are still isolated in stable patients, indicating chronic colonisation. This shows the loss of sterility in the respiratory tract due failure of the immune system [26].

Determining the pathogenesis of bronchiectasis may be beneficial for the management and treatment of the disease [23]. Two individual studies have shown a diagnosis of aetiology to be beneficial in treatment of 15% [23] and 37% of bronchiectasis patients [35]. Treatment is particularly effective in those with congenital conditions.

### **1.1.6 Management of Bronchiectasis**

Currently there is no standardized option for the treatment of bronchiectasis. Treatment plans for bronchiectasis are often an extrapolation from the treatment of other respiratory conditions, for example, CF, COPD and pneumonia [19, 42]. Planning treatment is often difficult due to the heterogenic nature of bronchiectasis. There are very diverse phenotypes, some individuals will present with very few symptoms, whereas others experience symptoms daily [22]. There

is a need for understanding the heterogenic nature of bronchiectasis in the development of new treatments [43].

Bronchiectasis can also be either focal or diffuse depending on the number of lobes and lung segments that are affected [3]. Bronchiectasis requires ongoing long-term management of the condition, aimed at reducing the frequency of exacerbations thereby removing the potential for further lung damage. The goal of treatment is to increase the overall quality of life for bronchiectasis sufferers [13].

Treatment can only commence once diagnosis is confirmed by HRCT scan. Then determination of aetiology and severity of disease needs to be made. Factors often used to assess severity include, lung function decline (%FEV<sub>1</sub>), exacerbation frequency and changes in sputum colour, volume or microbiology [16, 44]. Health-related quality of life (HrQoL) questionnaires, for example, St George's Respiratory Questionnaire and Leicester Cough Questionnaire, are also commonly used [4].

Lung function decline is typically monitored by measuring the forced expiratory volume in one second (%FEV<sub>1</sub>) of patients [3]. Studies have shown an estimated mean decline in %FEV<sub>1</sub> of 33-55mL per year in patients with non-cystic fibrosis bronchiectasis [20, 35, 41, 44]. No long-term treatment has had any impact so far on the declining FEV<sub>1</sub> in bronchiectasis.

Current treatment is based on a combination of antibiotics, anti-inflammatories, chest physical therapy, and surgery [45]. Antibiotics are usually administered upon admittance for an exacerbation. There has been a move towards the use of oral antibiotics (eg. nebulised aminoglycoside) as they are more specific, rather than intravenous antibiotics [45]. Recommendations exist for a more individualized approach for patients, by culturing the sputum and selecting antibiotics based on results [22]. But not all patients have microorganisms isolated from their sputum [13]. Very limited randomised controlled trials exist for antibiotics in bronchiectasis [45].

There is the possibility for the use of prophylactic antibiotics, particularly for stable bronchiectasis patients. This aims to reduce their bacterial burden; thereby breaking the 'vicious cycle' [45]. Very limited evidence is available to support this approach [19]. Using prophylactic oral antibiotics has been shown to reduce the effectiveness of oral antibiotics during an exacerbation [16].

Anti-inflammatories (eg. inhaled corticosteroids or oral macrolides) help to reduce the amount of structural damage done to airways, by suppressing the inflammatory response [13, 22]. A recent randomised double-blind trial was undertaken in New Zealand using the macrolide antibiotic Azithromycin [46]. This drug works via its anti-inflammatory and immunomodulatory properties. Findings showed an exacerbation rate of 0.59 per patient in the Azithromycin group and 1.57 per patient in the placebo group. This data therefore indicates the usefulness of Azithromycin in reducing frequency of exacerbations. Care though needs to be taken to prevent the development of macrolide resistance [46].

Commonly used is chest physical therapy for clearance of sputum from the airways. Surgery or lung transplant are reserved for very end-stage bronchiectasis; therefore only considered if bronchiectasis is focal, resistant or no longer responding to aggressive medical therapy [4, 45].

Significantly more research is needed in the development of suitable long-term treatments for bronchiectasis individuals. The heterogenic nature of bronchiectasis suggest that treatment should become more individualized, for example, culturing sputum and selecting antibiotics based on microbes isolated [43]. Additionally, it would be beneficial if specific biomarkers were identified to guide therapy during exacerbations [4]. Prevention and early diagnosis should be the focus of future treatment options [9].

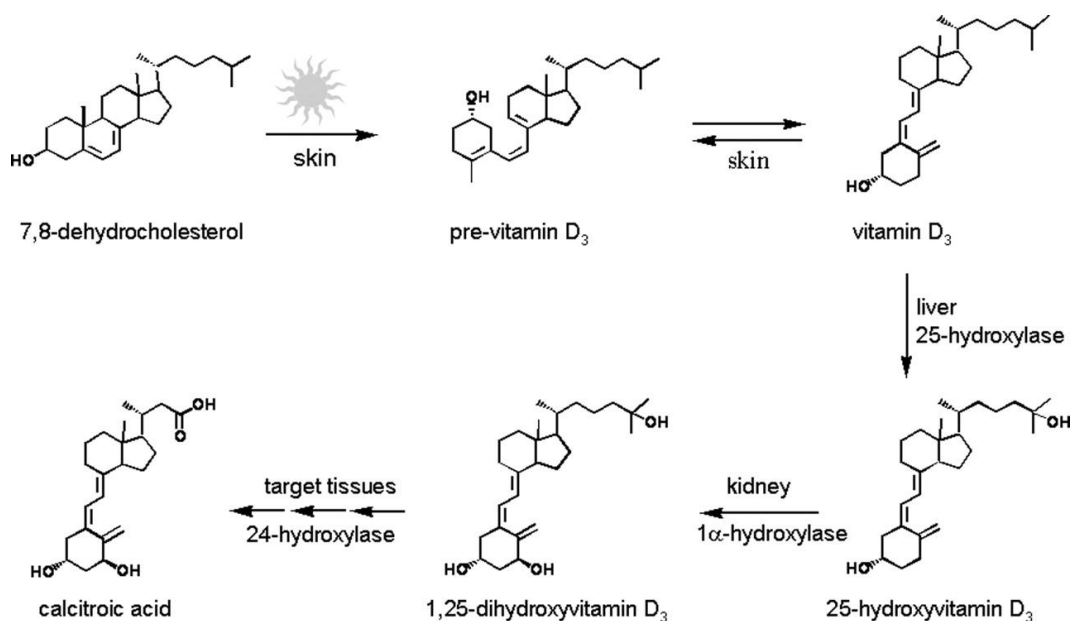
## 1.2 Vitamin D

### 1.2.1 Physiology

Vitamin D is classified within the family of secosteroid hormones [47]. Vitamin D exists in two main forms: cholecalciferol (vitamin D<sub>3</sub>) and ergocalciferol (vitamin D<sub>2</sub>) [47, 48]. The predominant source of vitamin D is from sun exposure (90%) and the minority from diet (5-10%), either through an oily fish diet or supplementation [49]. Limited foods naturally contain vitamin D; therefore many foods are now fortified yet currently not in New Zealand [50].

The two main forms of vitamin D are biologically inactive and will undergo two sequential hydroxylation steps to become active [51, 52]. Vitamin D<sub>3</sub> is produced by 7-dehydrocholesterol by thermal isomerization upon exposure to UVB radiation on the skin [53]. Vitamin D<sub>3</sub> can then enter the circulation where it is bound to vitamin D binding protein (DBP) for transportation. DBP is the highest affinity binder of the vitamin D analogues; albumin acts as a low affinity carrier [54, 55].

Hydroxylation occurs in the liver, at position 25 of vitamin D<sub>3</sub>, by the enzyme 25-hydroxylase (CYP27A1 or CYP2R1) to form 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) [56]. This is the major circulating form of vitamin D, used to determine vitamin D status [49]. Further hydroxylation occurs in the kidney by 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase (CYP27B1) to form 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>, calcitriol); the active vitamin D metabolite (Figure 3) [57]. This renal production is tightly regulated by levels of parathyroid hormone (PTH), calcium and phosphorus [58, 59]. There are over 50 known vitamin D metabolites [60].



**Figure 3** The synthesis, bioactivation and inactivation of vitamin D<sub>3</sub>, as published by Dusso, 2005 [61]

### 1.2.2 Classical Function

The association between sunlight and skeletal health has long been known. Most notably at the beginning of the twentieth century, when there was widespread incidence of children with nutritional rickets and growth retardation. This arose predominantly during the industrial revolution in Europe, where the excessive pollution greatly reduced children's exposure to sunlight. The earliest description of rickets was by Whistler in the 1600s, and later on by Glisson in 1951 [59]. Rickets is the result of insufficient calcium intake that effects bone development causing weakened, deformed bones and muscle spasms [59, 62]. The use of UV light for the treatment of rickets was commonly used, as demonstrated by Shiadecki in 1822, and later on by Hess and Unger [59, 62, 63]. But it was sometime before the molecule vitamin D was identified in this association [64].

The primary role of vitamin D is in bone homeostasis via the renal production of 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D). Maintaining calcium-phosphate homeostasis is vital for preserving bone health. Calcium is required to be maintained within a narrow concentration range, otherwise bone reabsorption results, leading to bone mineralisation [49, 65].

Vitamin D deficiency (VDD) therefore has numerous effects on skeletal health. Serious VDD traditionally results in rickets, osteomalacia or osteoporosis [48, 65]. When vitamin is insufficient it can result in hypovitaminosis D, causing reduced calcium absorption leading to secondary hyperparathyroidism. Ultimately causing accelerated loss of bone and increases the risk of osteoporotic fracture [48, 65].

The production of renal  $1,25(\text{OH})_2\text{D}$  is stimulated via the actions of PTH [66]. Negative feedback from the serum levels of calcium and phosphate are also involved [48]. The classical targets of renal  $1,25(\text{OH})_2\text{D}$  is the bone, kidneys and intestines [48]. This active form,  $1,25(\text{OH})_2\text{D}$ , can be further catabolized in the kidneys. This only occurs when  $1,25(\text{OH})_2\text{D}$  levels are sufficient. This step is carried out by the 24-hydroxylase enzyme (CYP24A) to form inactive  $24,25\text{-dihydroxyvitamin D}_3$  via a negative feedback loop (Figure 3). Active  $1,25(\text{OH})_2\text{D}_3$  induces expression of CYP24A, resulting in hydroxylation of  $25(\text{OH})\text{D}$  or  $1,25(\text{OH})_2\text{D}$  at position 24, producing biologically inactive metabolites that can be excreted [61, 66].

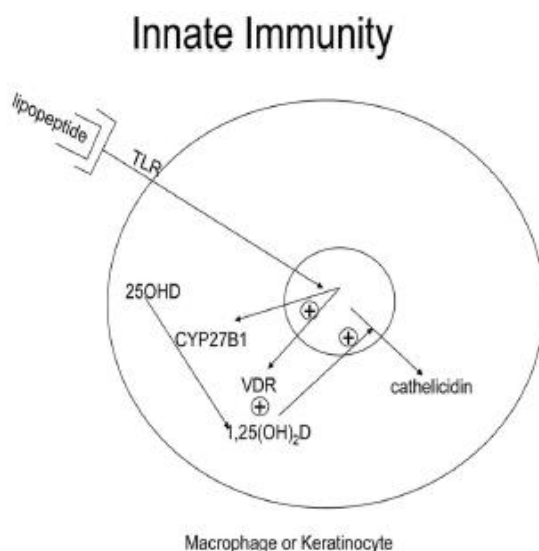
### **1.2.3 Non-Classical Function**

Further research has revealed that the hormonal form  $1,25(\text{OH})_2\text{D}$  has pleiotropic effects [67]. In addition to its classical endocrine action, both autocrine and paracrine pathways have been discovered [68]. Vitamin D is also involved in the regulation of cell growth, angiogenesis, apoptosis, and differentiation. Furthermore, as a chemopreventative agent in a number of cell types [67].

It is now known that  $25(\text{OH})\text{D}$  can be utilised by a multitude of cell types to locally synthesise the hormonal form,  $1,25(\text{OH})_2\text{D}$  [67]. The enzyme  $25\text{-hydroxyvitamin D-}1\alpha\text{-hydroxylase}$  (CYP27B1), the vitamin D activating enzyme, is expressed by many peripheral tissues especially within the immune system [69, 70]. The vitamin D receptor (VDR) has also been found in numerous extra-renal tissues and immune cells [71]. These vital components are found in most immune cells including, T cells [72], B cells [73], neutrophils [74], monocytes, macrophages [75] and dendritic cells [76]. Monocytes and macrophages have

been shown *in vitro* to upregulate both VDR and CYP27B1 (Figure 4), upon exposure to a bacterial lipopolysaccharide (LPS) or the microorganism *Mycobacterium tuberculosis* via TLR2/1 [77]. The potential therefore exists for the local production of 1,25(OH)<sub>2</sub>D.

Bronchial epithelium and immune cells are capable of converting the circulating precursor 25(OH)D<sub>3</sub> to local active 1,25(OH)<sub>2</sub>D<sub>3</sub> [52]. It has been demonstrated that these epithelial cells of the respiratory tract are capable of having constitutively high levels of 1 $\alpha$ -hydroxylase (CYP27B1) and low levels of the inactivating 24-hydroxylase. These elements therefore enable these cells to create a microenvironment with high active levels of vitamin D [52]. This allows localized synthesis at sites prone to inflammation [69].



**Figure 4** 1,25(OH)<sub>2</sub>D stimulates the innate immune system. TLR's trigger the macrophage to increase VDR and CYP27B1 activation, ultimately causing synthesis of 1,25(OH)<sub>2</sub>D.

1,25(OH)<sub>2</sub>D is then able to induce cathelicidin production, as published by Bikle 2009 [78]

The nuclear VDR is a 1,25(OH)<sub>2</sub>D-activated transcription factor [61, 67]. Active 1,25(OH)<sub>2</sub>D enters the target cells and binds to VDR in the cytoplasm, where it then enters the nucleus to form a heterodimer with the retinoid X receptor (RXR) [47, 79]. This complex can then bind to vitamin D response elements (VDRE's) to regulate transcription of target genes [47, 61, 66]. VDRE's are made up of PuG (G/T) TCA repeating motifs divided by three base pairs or reverse repeats

separated by six base pairs [66]. The fact that VDRE's are highly conserved in humans and primates and not found in non-primate animals, suggests that is recent significant adaptation [80]. 1,25(OH)<sub>2</sub>D<sub>3</sub> has been shown to regulate over 900 genes in a microarray study [81].

The mechanisms for the regulation of non-renal 1,25(OH)<sub>2</sub>D is poorly understood, and is possibly under the control of local factors, for example, cytokines or growth factors [48, 82]. Local production is not regulated by PTH like renal forms, so 1,25(OH)<sub>2</sub>D concentration is dependent on its substrate 25(OH)D concentration [82].

The importance of vitamin D on health has been increasingly acknowledged over the last decade. This is primarily due to the recognition of vitamin D as a modulator of numerous other biological responses, not just its traditional effects on skeletal health [67]. A considerable number of chronic and immune-mediated diseases can now be associated with vitamin D insufficiency [49]. The active form 1,25(OH)<sub>2</sub>D is now known to have immunomodulatory effects due to its synthesis within epithelia and immune cells, and via its downstream effects on target genes.

### **1.2.3.1 Immunomodulation of the Innate Immune System**

The innate immune system is an important non-specific first line of defence against infections [83]. Epithelial cells act as a protective physical barrier between the tissues and environment [83-85]. This barrier is especially important in the skin, gut, urinary and respiratory tract, where the innate immune system works to eliminate foreign organisms [83, 85]. Lungs and airways are constantly exposed to an array of inhaled pathogens and therefore require an effective innate immune system to ward against infection [84]. The active form, 1,25(OH)<sub>2</sub>D is potentially an important contribution to host defense of the lung [52].

The hormonal form 1,25(OH)<sub>2</sub>D is now known to have potent immunomodulatory effects [61, 67]. The initial link between vitamin D and immunomodulatory effects came as a result of an association between vitamin D insufficiency and the occurrence of *Mycobacterium tuberculosis* [86]. The presence of vitamin D



greatly increased killing of the mycobacterium, via upregulation of cathelicidin in macrophages upon exposure to a mycobacterial ligand (Figure 4)[77].

These immunomodulatory effects arise via  $1,25(\text{OH})_2\text{D}$  genomic actions. *In vitro* studies have shown that  $1,25(\text{OH})_2\text{D}_3$  is involved in the direct regulation of antimicrobial peptides in numerous tissue types and cell lines [87, 88]. Both cathelicidin and  $\beta$ -defensin 2 (hBD-2) promoters contain VDRE's [88].

### 1.2.3.2 Antimicrobial Peptides

Antimicrobial peptides (AMP's) are a vital component of the vertebrate innate immune system and are regarded as an 'evolutionary ancient weapon' [89].

AMP's are defined as cationic polypeptides consisting of less than 100 amino acids (generally 3-5kDa) that possess antimicrobial activity against bacteria ( $\text{G}^+$  and  $\text{G}^-$ ), enveloped viruses and fungi, under physiological conditions [84, 90]. AMP's are generally widespread and diverse in their nature. AMP's have been isolated from many body secretions, as well as in numerous cell types. Certain parts of the amino acid sequence are highly conserved [84], particularly those involved in the translation, secretion and transport [89]. AMP's are recently of interest as they show potential antimicrobial effects against antibiotic resistant bacterial strains [83], for example, *Pseudomonas aeruginosa* [88].

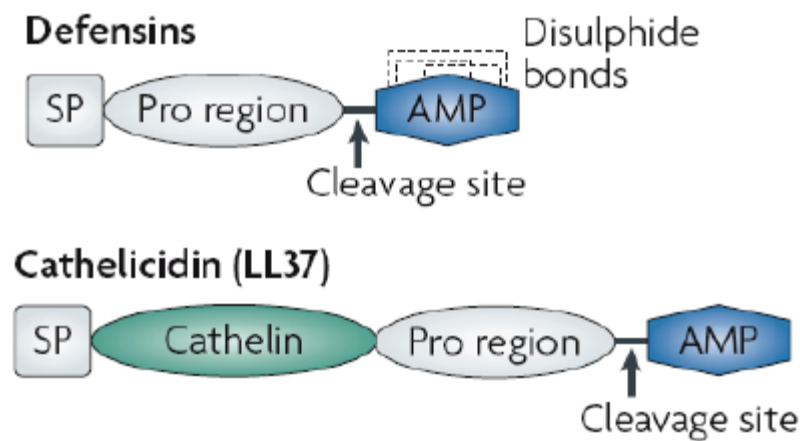
The broad spectrum antimicrobial function of these peptides comes from their ability to permeate and disrupt the membrane of target cells [83, 85, 89]. The outer bacterial membranes are generally negatively charged which provides an electrostatic attraction with the cationic peptide [83, 85]. AMP's are also amphipathic in nature, possessing both hydrophilic and hydrophobic parts, which aids in membrane destabilization [83, 84]. Layering of AMP's on the membrane leads to pore formation [91]. Eukaryotic cells are protected against AMP action due to the presence of cholesterol in the cell membrane [85]. Pathogen-associated molecular patterns (PAMP's), for example, LPS, flagellin, or viral proteins, can stimulate pathogen recognition receptors (PRP's) on cell membranes [92]. TLR's are a class of PRP's which when stimulated can then induce the CYP27B1 enzyme (Figure 4) [93].

There are numerous AMP families divided up based on their secondary protein structure. Defensins and cathelicidins are the two predominant AMP families in humans [84]. Defensins and cathelicidins have been found to have some synergistic actions [94, 95].

The cathelicidins do not contain any cysteine residues allowing them to form a linear  $\alpha$ -helical formation. The cathelicidin (CAMP) gene is located on chromosome 3 and is made up of four exons, only exon 4 is responsible for the antimicrobial properties [96]. The CAMP gene product is a pre-propeptide and becomes a propeptide once the signal sequence has been removed. The propeptide undergoes proteolytic processing (by proteinase 3 in neutrophils) to remove the 'cathelin-like domain', to release the mature active peptide (LL-37) [97]. But there is only one cathelicidin found in humans, hCAP-18/LL-37 [84]. Cathelicidin is found in the secondary granules of both neutrophils and leucocytes [98].

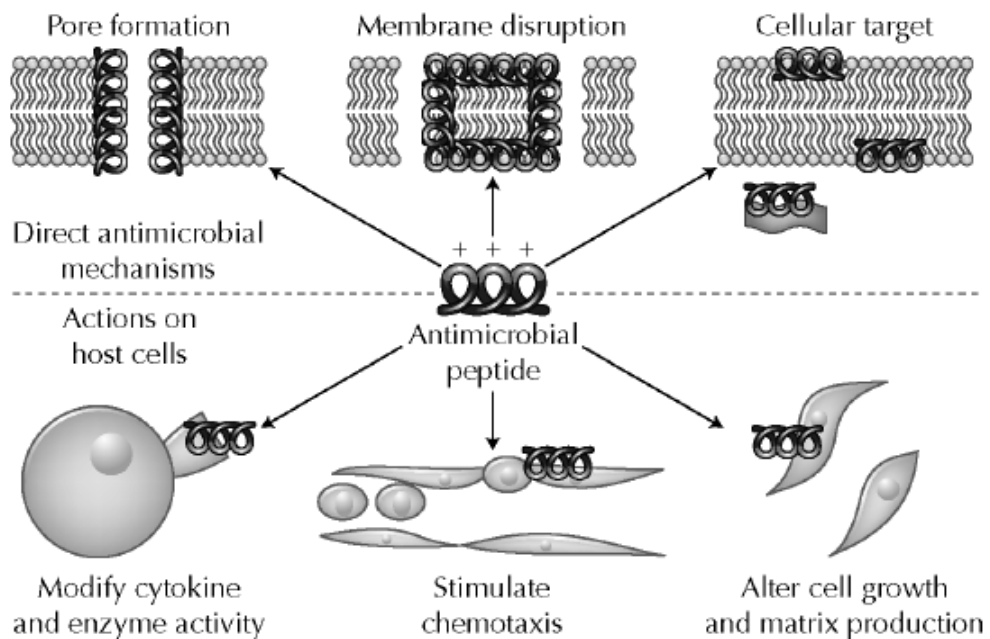
Defensins are prevalent in humans where two major families exist,  $\alpha$ - and  $\beta$ -defensins [84]. Humans possess 6  $\alpha$ -defensins and 4  $\beta$ -defensins genes located in a cluster on chromosome 8 [90]. The families are split based on differences in the pairing of the six cysteine residues that make up the three disulphide bridge [84, 90]. These three disulphide bridges cause the peptide to fold into a  $\beta$ -sheet structure [83]. The  $\beta$ -defensins are typically larger in size than  $\alpha$ -defensins.

$\beta$ -defensins are more widely distributed than  $\alpha$ -defensins; the major synthesis of  $\beta$ -defensins is by epithelial cells. The expression of  $\beta$ -defensins 2-4 within epithelial cells has been revealed to be inducible [84], compared to human  $\beta$ -defensin 1 which is continuously expressed [99]. hBD-2 can also be synthesized in monocytes, macrophages and dendritic cells [84]. The active defensin peptide is formed in a similar fashion to cathelicidin, with the signalling molecule and pro-region being removed to release the mature AMP (Figure 5) [100]. No single mechanisms of defensins antimicrobial properties has been identified but thought to be similar to cathelicidins mode of action.



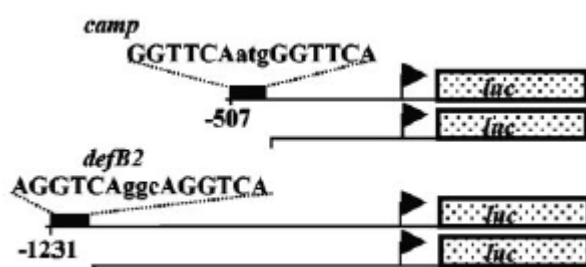
**Figure 5 Structural similarities of cathelicidin and defensin precursors, as published by Kolls et al., 2008 [100]**

All of the cathelicidins and defensins have numerous other immune regulatory functions in addition to their antimicrobial activity (Figure 6), both can act as chemoattractants [84, 94]. In addition to its antimicrobial properties as part of the innate immune system, LL-37 also acts as a signalling molecule helping to regulate the adaptive immune system [101]. Cathelicidin can act as a chemoattractant to recruit immune cells to sites of infection and also mediates proinflammatory actions [96, 102].



**Figure 6 All known biological functions of mammalian antimicrobial peptides that function in innate immune system, as published by Gallo & Nizet, 2003 [94]**

Importantly, both defensins and cathelicidins are found in airway and lung epithelium, as well as lung secretions [84, 103]. Their expression is generally triggered mainly by exposure to microbial products, for example, LPS via toll-like receptors [88], but also to cytokines and growth factors [84]. 1,25(OH)<sub>2</sub>D<sub>3</sub> is now recognised as a direct regulator of AMP expression. 1,25(OH)<sub>2</sub>D<sub>3</sub> alone causes strong induction of CAMP expression but modest effect on hBD2 expression [88]. The genes of both cathelicidin and hBD-2 contain VDRE's in their promoters; 507bp and 1231bp upstream of start codon respectively (Figure 7) [88]. The VDRE in the CAMP gene is embedded in an Alu repeat transposable element (primate specific) [80].



**Figure 7** The location of the *camp* and hBD-2 gene's VDRE's in the promoter region, as published by Wang *et al.*, 2004 [88]

### 1.2.3.3 Vitamin D Regulation of Antimicrobial Peptides Studies

Multiple regulators have been identified in the expression of LL-37 and hBD-2. One of which is the hormonally active form of vitamin D, 1,25(OH)<sub>2</sub>D<sub>3</sub>, which has been shown to regulate the expression of hBD-2 and LL-37 in a variety of cell types [87, 88]. Altered expression of AMP's occurs during inflammation and infection. Increased expression is commonly seen in individuals with inflammatory or infectious diseases [84].

Wang *et al.*, 2004 study tested the expression of the CAMP and hBD-2 genes *in vitro* in specific human cell types. Both CAMP and hBD-2 genes contain VDRE's in their promoter region. The treatment of these cell types with 1,25(OH)<sub>2</sub>D<sub>3</sub> caused the induction of LL-37 expression, observed by the mRNA upregulation in all cell types tested. These cells included keratinocytes, neutrophils, monocytes,

squamous carcinoma cells (SCC25), lung adenocarcinoma (Calu-3), and myelomonocytic cells (U937). The induced LL-37 was then shown to have antimicrobial properties against *Pseudomonas aeruginosa*. The hBD-2 expression though was only enhanced in certain epithelial cell types, SCC25 and keratinocytes. This is consistent with hBD-2 only being expressed in epithelial cells [88].

Other *in vitro* research further supports the upregulation of CAMP expression by  $1,25(\text{OH})_2\text{D}_3$  in other human cell lines and primary cultures including, urogenital cells, epithelial cells, neutrophils, lung [88], bronchial [104], colon, macrophages, keratinocytes and myeloid cell lines [87].

Various association studies have revealed contrasting results for effects of vitamin D on antimicrobial concentrations *in vivo*. Jeng *et al.*, 2009 research found a weak, but statistically significant positive association, between serum  $25(\text{OH})\text{D}$  and LL-37 in critically ill patients, with and without sepsis [105]. Another study has shown that serum hCAP18 is associated with increased mortality from infection in those with end-stage renal disease. No association was found between  $25(\text{OH})\text{D}$  and hCAP18. But interestingly a borderline association ( $p=0.053$ ) between serum  $1,25(\text{OH})_2\text{D}$  and hCAP18 was found [106].

Respiratory studies have looked at the levels of vitamin D, cathelicidin and hBD-2 in community acquired pneumonia. Severe VDD ( $<30\text{nmol/L}$ ) was associated with the increased 30-day mortality rate. But no correlation was found with  $25(\text{OH})\text{D}$  serum levels and antimicrobial levels [107]. Similarly, in a recent bronchiectasis study it was hypothesized that LL-37 levels in the airways would decrease in vitamin D deficient individuals. The results showed the opposite, with the highest LL-37 levels being found in the vitamin D deficient group. This suggests that vitamin D effects *in vivo* have little effect on LL-37 level [43]. Currently limited evidence supports the action of  $1,25(\text{OH})_2\text{D}$  on antimicrobial levels *in vivo*.

## 1.3 Vitamin D Deficiency

The occurrence of suboptimal vitamin D levels is widespread, with an estimated one billion individuals globally being estimated as vitamin D deficient [43]. The mean concentration of 25(OH)D in New Zealand adults (over 15 years) is 50nmol/L (n=2,946) [50]; identical mean to the UK National Diet and Nutrition Survey (2000-2001) by Ruston et al., 2004 [108]. In total 3% of the New Zealand sample population is regarded as deficient (<17.5nmol/L) and 48% insufficient (<50nmol/L); 84% (<80nmol/L) were insufficient based on different cut-offs points used [109] for vitamin D insufficiency [50]. Lower vitamin D levels were reported in Maori (mean=42nmol/L) and Pacific (mean=37nmol/L) [50]. UK nationwide study reported that 50% adults vitamin D insufficient and 16% severely deficient [108]. Higher rates have been reported in Scotland [110].

Risk factors of VDD include latitude, lack of sunlight, sunscreen use, poor diet, obesity, premature birth, gender, ethnicity, and age [48, 49]. These risk factors all prevent UVB radiation from reaching the skin. UVB radiation (300-325nm) is required for the thermal isomerization of precursor 7-dehydrocholesterol which varies due to latitude and seasonality [111]. The amount of UVB photons getting to earth relies on the sun's zenith angle [49]. New Zealand is located at a high latitude (35-47°C) meaning that radiation intensity is inadequate for long periods of the year [50]. In New Zealand the most significant determinants are ethnicity and season [112]. Darker skin has higher concentrations of melanin, which is known to decrease the synthesis of vitamin D from its precursor 7-dehydrocholesterol [65, 113]. Age is also an identified risk factor as there is a reduced concentration of the vitamin D precursor, 7-dehydrocholesterol, within the skin [65].

### 1.3.1 Measuring Vitamin D Status

Serum 25(OH)D levels are the best short-term measure of vitamin D status. 25(OH)D is the primary form of circulating vitamin D metabolite [60, 114], with a half-life of approximately 3 weeks [115]. This makes it the most accurate metabolite for measuring total vitamin D status [51]. The production of 25(OH)D is not tightly regulated like the hormonal form of vitamin D (1,25(OH)<sub>2</sub>D),

therefore only dependent on its substrates concentration [82]. 1,25(OH)<sub>2</sub>D is typically not used in the assessment of vitamin D status as it has a relatively short half-life (4 hours) and is tightly regulated by calcium homeostasis [60].

Determining vitamin D status is complex due to the number of vitamin D metabolites, particularly the two forms vitamin D<sub>2</sub> and vitamin D<sub>3</sub> [60], as well as the free vitamin D levels [60]. Approximately 99% of vitamin D metabolites are bound within the blood [116], this may affect their transportation into airways [117]. DBP is the predominant serum carrier of vitamin D and its metabolites [54, 60]. Single nucleotide polymorphisms in genes involved in vitamin D transport and metabolism may be a contributing factor in VDD, for example, DBP and VDR. Polymorphisms may be affecting the bioavailability of circulating vitamin D [118].

Determining vitamin D status is difficult as there is no unified consensus on the required or optimal serum concentration of 25(OH)D for optimal health [47, 49, 112]. A number of studies suggest a level of less than 50nmol/L (<20ng/mL) of 25(OH)D for VDD, insufficient level of 50-75nmol/L (<20-30ng/mL) [49, 112, 119] and sufficient levels of 75-80nmol/L (30-32ng/mL) [120-122]. Vitamin D intoxication is very rare and occurs at 25(OH)D levels above 375nmol/L (150ng/mL) [49].

To further complicate matters, suggestions have been made the levels required for bone health may vary from that required for its other functions. There is an inverse relationship between 25(OH)D concentrations and levels of circulating PTH until reaches 30ng/mL where PTH plateaus [66, 121, 122]. The current status quo level of 50nmol/L serum level of 25(OH)D may not be enough for the prevention of other diseases. The serum 25(OH)D level may in fact need to be higher, for example, 75-80nmol/L [123] or 100-150nmol/L [93]. The evidence to support this is currently limited and further research is needed. The levels required for bone health may vary from that required for its other functions.

### 1.3.2 Treatment of Vitamin D Deficiency

Supplementation for VDD is currently being reinvestigated. Current supplementation guidelines (Food and Nutrition Board) suggest 200 IU/day for those aged under 50 years; 400 IU/day for those between 50-70 years; and 600 IU/day for those over 70 years [65]. Further research is needed to determine the best dosing strategy for the treatment of VDD [124].

Often no observed change in serum 25(OH)D is seen after a vitamin D dosing regimen [124]. Emerging evidence is indicating that cholecalciferol (vitamin D<sub>3</sub>) may be a more efficacious form [124, 125]. A recent study has shown that the potency of vitamin D<sub>2</sub> is only one-third of the potency of vitamin D<sub>3</sub>, achieved by mapping the time-course of 25(OH)D<sub>3</sub> after a single dose [124]. Potentially this is due to DBP having a higher affinity for vitamin D<sub>3</sub> leading to it having a longer circulating half-life [124]. More research is needed to come up with a standardized way of measuring and dosing VDD.

## 1.4 Vitamin D Deficiency and Chronic Disease

The hormonal form 1,25(OH)<sub>2</sub>D due to its pleiotropic actions has been associated with decreased risk of many chronic diseases [49]. The actions of vitamin D have been implicated with various autoimmune, cardiovascular, respiratory [43], metabolic and infectious diseases [49]. Also, associated with certain cancers [49, 126-128], diabetes [129], muscle weakness, hypertension and neurological conditions (eg. multiple sclerosis [130]) [49].

Vitamin D was unknowingly used to treat tuberculosis, where it was found to be the active component of Cod Liver Oil which was a commonly used treatment. UV light was also commonly used to treat forms of TB during the mid-1900. VDD is a risk factor for tuberculosis [131]. One study has shown that exposure of *Mycobacterium tuberculosis* to TLR2/1 caused increased expression of VDR and CYP27B1 in monocytes [77]. 1,25(OH)<sub>2</sub>D treatment resulted in killing of *Mycobacterium tuberculosis* in cultured human macrophages [132].



VDD has also been linked to an enhanced risk of respiratory infections [43, 133]. A VDD mouse model shows changes in lung structure and a decreased lung function [134]. Associations have been found between low levels of vitamin D and the occurrence of respiratory tract infections in both Finnish [135] and American populations [136]. Low vitamin D levels have been found in patients with COPD and found to correlate with COPD severity [133]. Another study has looked at the vitamin D levels and community-acquired pneumonia. Severe VDD ( $<30\text{nmol/L}$ ) was associated with the increased 30-day mortality rate in these patients [107].

#### **1.4.1 Vitamin D Deficiency and Bronchiectasis**

Chalmers *et al.*, 2013 paper provides the first study providing data on the association of VDD in bronchiectasis and disease severity [43]. VDD has been previously associated with a risk of respiratory infections; now including bronchiectasis. This research measured the concentration of 25(OH)D in 402 stable non-cystic fibrosis bronchiectasis patients. A total of 50% deemed vitamin D deficient ( $<25\text{nmol/L}$ ), 43% insufficient ( $25\text{-}74\text{nmol/L}$ ) and 7% sufficient ( $>75\text{nmol/L}$ ), compared to 12% deficient in control [43].

VDD is found to be associated with several markers of bronchiectasis disease severity, particularly chronic bacterial colonisation. A total of 21.4% of vitamin D deficient bronchiectasis patients were chronically colonised by *Pseudomonas aeruginosa*, compared to only 10.4% of insufficient patients and 3.6% of sufficient patients. Those classed as vitamin D deficient exhibited lower %FEV<sub>1</sub>, accelerated decline in lung function, higher frequency of exacerbations, more severe exacerbations, worse health-related quality of life (SGRQ and LCQ) and higher levels of sputum inflammatory markers [43].

More evidence is needed to determine exact causality. But these results suggest that a randomised controlled trial of vitamin D replacement therapy may be beneficial to determine if any changes occur in clinical outcomes [43]. Important to note that VDD most often results from lack of sunlight and that having a respiratory condition often leads to reduced outdoor activities [43, 49, 108, 133].

## 1.5 Inflammatory Markers

Inflammatory markers are biomarkers in the blood that indicate the amount of inflammation occurring during an infection. This can then potentially be used to guide antibiotic therapy and aid in prognosis [137, 138]. Bronchiectasis patients are susceptible to reoccurring lower respiratory tract infections, which produce an inflammatory response [5].

There are indications that stable patients with non-cystic fibrosis bronchiectasis actually have consistently elevated systemic markers of inflammation, including C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) [139]. Due to chronic infection, bronchiectasis patients continue to have active local and systemic inflammation even during stable phase [137, 140]. Currently no specific inflammatory marker suitable for bronchiectasis has been found [138].

Procalcitonin (PCT) is a relatively new, yet still controversial, marker of inflammation that proved useful in the treatment of some conditions [141-143]. PCT is the precursor peptide of calcitonin (a hormone), that is increased in response to bacterial infections. Is typically secreted by the body's parenchymal cells and is produced in the C cells of the thyroid gland [144]. In response to a period of inflammation should see an elevated serum level. Normally PCT levels are very low ( $<0.05\text{ng/mL}$ ), as PCT is not constitutively released into the blood, and only mildly elevated in viral infections (up to  $2\text{ ng/mL}$ ) [145]. But in severe bacterial infections serum PCT can reach over  $700\text{ng/mL}$  [145].

A number of studies have indicated the usefulness of PCT in separating infections that are viral or bacterial [146]. CRP is regarded as a nonspecific inflammatory marker of systemic inflammation. PCT is considered to be potentially more sensitive and specific, than the more commonly used CRP in defining bacterial infections over systemic infections [147]. PCT also peaks sooner after infection (8 hours) compared to traditional CRP (36 hours) [147].

PCT has proved useful in reducing the amount of antibiotics administrated but with no change in clinical outcome [141-143]. Reduction in antibiotic use shown

in sepsis (down 23%) [143], in LTRI, COPD, community-acquired pneumonia (reduction of 55% antibiotic days) [148, 149] and in COPD [142]. Using PCT may provide additional prognostic information for patients that are at high risk, as indicated in community-acquired pneumonia [150]. These studies are based on specific cut-off points: 0.1ug/L indicates no bacterial infection/no antibiotics required, 0.1-0.25ug/L bacterial infection unlikely/only antibiotics if high risk patient, 0.25-0.5ug/L indicates possible bacteria/requires antibiotics and >0.5ug/L strong bacterial infection/antibiotics needed [148, 149].

To date, Loebinger *et al.*, 2008 study is the only one on PCT in bronchiectasis patients. PCT may be able to guide in bronchiectasis exacerbation treatment, enabling the length of the antibiotic course to be personalized [137, 138]. This investigation found that patients admitted with an infective exacerbation had quite low or negative PCT levels (0.055ug/L) [138], similar to another COPD study (0.096ug/L) [142]. Pneumonia typically has higher concentrations (0.5ug/L) [150]. This may be due to the fact that in COPD and bronchiectasis inflammation is more localized [138]. No published research to date has investigated the levels of PCT in sputum.

## **1.6 Study Design**

This research is a sub-component of a multi-centre clinical trial investigating the usefulness of Tiotropium in the treatment of non-cystic fibrosis bronchiectasis. Tiotropium is a novel inhaled anticholinergic drug that only requires once a day dosage. The prolonged bronchodilatory effect of Tiotropium comes from its prolonged antagonism of muscarinic receptors. The use of Tiotropium has been linked to improvements in airflow in COPD [151, 152].

The clinical trial was run as a double-blind crossover study. For each half of the 54 weeks patients would receive either the drug or placebo; with serum and sputum samples being collected at Weeks 1, 26, 30 and 54. These samples allowed an investigation into the role of vitamin D, cathelicidin, hBD-2 and PCT in stable bronchiectasis patients. Using enzyme-linked immunosorbent assay (ELISA). These concentrations will be analysed for any association using

predetermined cut-off points or tertiles, and Spearman's non-parametric correlations.

### 1.6.1 Vitamin D Analytes

Vitamin D status is determined by measuring serum 25(OH)D levels. 25(OH)D is the predominant circulating vitamin D metabolite that provides the best measure of vitamin D status. The cut-off points that will be used are: <30nmol/L severe VDD, <50nmol/L VDD, 50-75nmol/L insufficient (<20-30ng/mL) [49, 112, 119] and >75nmol/L sufficient [120-122].

Vitamin D is now known to directly regulate specific target genes. *In vitro* studies have shown the ability of 1,25(OH)<sub>2</sub>D<sub>3</sub> to directly regulate the expression of various AMP's, including cathelicidin. But previous studies of community acquired pneumonia, have failed to show any correlation between vitamin D concentrations with the AMP's (cathelicidin and hBD-2) [107]. Potentially the reason for this lack of association could be due to serum levels not accurately representing localized tissue levels. In fact there may be an unequal distribution of vitamin D, making the analysis of vitamin D metabolites in sputum important. Very little is known about the concentrations of vitamin D metabolites in tissues and along mucosal surfaces. In human bronchoalveolar fluid (BAL), levels of vitamin D metabolites were typically very low [117].

1,25(OH)<sub>2</sub>D is typically not used in the assessment of vitamin D status, as has a relatively short half-life (4 hours) compared to 25(OH)D (3 weeks). There is a limited number of studies that have measured 1,25(OH)<sub>2</sub>D concentration in serum. There is no consensus on normal 1,25(OH)<sub>2</sub>D but renal 1,25(OH)<sub>2</sub>D is generally tightly regulated and not severely affected by fluctuating 25(OH)D serum levels. One Danish study reports an average 1,25(OH)<sub>2</sub>D level of 29.0pg/mL (+/- 9.5pg/mL) [153]. This is supported by an older study which revealed an average of 23.6pg/mL (+/-1.3pg/mL) in the control group [154].

But very little is understood about the local activation of vitamin D metabolites in these extra-renal tissues or mucosal surfaces. Or what their relationship is to the

circulating levels of cathelicidin and hBD-2. The effect of 1,25(OH)<sub>2</sub>D on expression of cathelicidin and hBD-2 has only been shown so far *in vitro* not *in vivo* [87, 88, 117]. Measuring the levels of both 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> in serum and sputum may reveal novel findings in their association with AMP levels *in vivo*. Tertiles will be used in the assessment of 1,25(OH)<sub>2</sub>D<sub>3</sub>, cathelicidin and hBD-2 concentrations, as no current consensus levels are available.

### **1.6.2 Procalcitonin**

PCT levels will be split into none, low, moderate or high. The low value will be those below 0.1ug/L (100pg/mL) which is commonly reported in normal healthy individuals and is associated with no bacterial infections. High PCT levels will be those above 0.5ug/L which indicates the presence of a strong bacterial infection [148, 149]. Moderate will be those that fall in between these values.

No published research to date has investigated the levels of PCT in sputum. It is estimated that PCT levels will be greater in sputum compared to serum levels. This is purely due to sputum being located at a site with a high density of bacteria and the presence of inflammatory cells. Unpublished data supports elevated PCT levels being present in sputum (2ng/mL) against serum (<0.5ng/mL) in bronchiectasis patients with acute exacerbations (Wong *et al.*, 2011).

## **1.7 Research Objectives**

The main objective of the study was to analyse the levels of vitamin D metabolites and antimicrobial peptides in stable bronchiectasis patients. The identification of any associations may provide an insight on the mechanisms involved in the development and progression of non-cystic fibrosis bronchiectasis. This may ultimately lead to improvement of the prevention and management of non-cystic fibrosis bronchiectasis.

### **1.7.1 Aims**

- 1) The primary aim of this research was to determine if differences exist between systemic (serum) and local (sputum) tissue concentrations of vitamin D metabolites, cathelicidin,  $\beta$ -defensin 2 and procalcitonin.
- 2) The secondary aim was to assess the serum vitamin D status in clinically stable bronchiectasis patients.
- 3) Lastly, to determine if there is an association between vitamin D status and the levels of antimicrobial peptides, cathelicidin and  $\beta$ -defensin 2 in individuals with bronchiectasis.

### **1.7.2 Hypotheses**

#### Hypothesis One

There will be differences between the localised and systemic concentrations of vitamin D, cathelicidin,  $\beta$ -defensin 2 and procalcitonin in non-cystic fibrosis bronchiectasis patients. A null hypothesis proposes that there will be no difference between localised and systemic levels of vitamin D, cathelicidin,  $\beta$ -defensin 2 and procalcitonin in bronchiectasis.

### Hypothesis Two

Vitamin D deficiency will be prevalent in stable non-cystic fibrosis bronchiectasis patients. A null hypothesis proposes that vitamin D deficiency will not be associated with the occurrence of bronchiectasis.

### Hypothesis Three

Non-cystic fibrosis bronchiectasis individuals with lower vitamin D levels will correspondingly have decreased levels of antimicrobial peptides, cathelicidin and  $\beta$ -defensin 2. A null hypothesis proposes that there will be no association between vitamin D concentration and antimicrobial peptide concentrations.

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# Chapter Two: Methodologies

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## 2.1 Sample Collection

This research was a subcomponent of a trial investigating the usefulness of Tiotropium in the treatment of bronchiectasis. Patients were being recruited from Waikato Hospital, Middlemore Hospital and Auckland Hospital. Ethical approval was obtained from the Northern Y Regional Ethics Committee (NTY/11/10/104). Participants selected for the trial were recruited from Middlemore and Auckland Hospitals. Written consent was obtained from each participant and their medical details were recorded.

The clinical trial was a double-blind crossover study run over a period of 54 weeks. These 54 weeks were divided up into two study periods. In one study period patients would receive Tiotropium and in the other a placebo based on their randomisation code. Whole blood and sputum samples were obtained at the initial visit (Visit One), 26 weeks (Visit Five), 30 weeks (Visit Six) and 54 weeks (Visit Nine). The visit's one and six serve as controls for their respective study period.

Peripheral whole blood and sputum was obtained at specific visits by a medical doctor. These were then processed at the hospital lab (2.2) to obtain the serum and sputum supernatant. These samples were then transported to the Molecular Genetics Laboratory at The University of Waikato (C.2.03) and stored at -80°C until required for ELISA analysis.

## 2.2 Sample Preparation

After blood and sputum samples have been collected both need to be processed to a more usable form for ELISA analysis. Serum and sputum supernatant is required for the measurement of PCT, cathelicidin,  $\beta$ -defensin 2, 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub>. Sputum supernatant is a more workable solution than pure sputum. All serum and sputum samples were stored at -80°C and then transferred to a



different lab to undergo analysis of PCT,  $\beta$ -defensin 2, cathelicidin,  $25(\text{OH})\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$ .

### 2.2.1 Serum Processing

The following procedure was undertaken at Middlemore Hospital and Auckland Hospital following collection of the patient's whole blood samples.

1. Collect whole blood sample.
2. Leave the blood to clot by leaving undisturbed at room temperature for approximately 15-30 minutes.
3. Centrifuge at 2500rpm for ten minutes in a refrigerated centrifuge.
4. The resultant supernatant is the serum. Remove carefully using a Pasteur pipette.
5. Samples stored in 1mL aliquots and stored at  $-80^\circ\text{C}$  until analysed.
6. Avoid repeated freeze-thaw cycles.

### 2.2.2 Sputum Processing

The following procedure was undertaken at Middlemore Hospital and Auckland Hospital following collection of the patient's sputum samples.

1. Weigh the sample and add twice the volume of 10% Sputolysin. (Note: results in a **1:3 dilution** of sputum; one part sputum and two parts sputolysin).
2. Mix with syringe and cannula, place cap on falcon tube and use parafilm to seal.
3. Put into shaking water bath for 30 minutes at  $37^\circ\text{C}$  (test if sample homogenous).
4. Mix gently and aspirate into a 10mL syringe using cannula.
5. Filter using  $50\mu\text{m}$  filter fabric inner and at a rate of 1 drop per second.

6. Gently mix and pipette 100 $\mu$ L of sample into Eppendorf tube. Add equal volume of Trypan blue.
7. Using Haemocytometer perform a total cell count.
8. Remaining sputum split into 1.6mL Eppendorf tubes and stored at -80°C; samples marked if screen-failed.

## **2.3 ELISA Kits**

Enzyme-linked immunoassay is commonly used to perform quantification of specified analytes. ELISA was used to determine the concentrations of PCT, cathelicidin, hBD-2, 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> in both serum and sputum. Each ELISA assay was carried out in duplicate and using internal standards for producing a standard curve, allowing the quantification of analyte concentration.

### **2.3.1 USCN Life Science ELISA Kit**

Commercially available sandwich enzyme immunoassay kits (USCN Life Science Inc., Wuhan, China) were used for independent *in vitro* measurement of PCT, cathelicidin and hBD-2. These kits were capable of measuring these analytes in human serum, plasma and other biological fluids. ELISA's were run according to manufacturer's protocol.

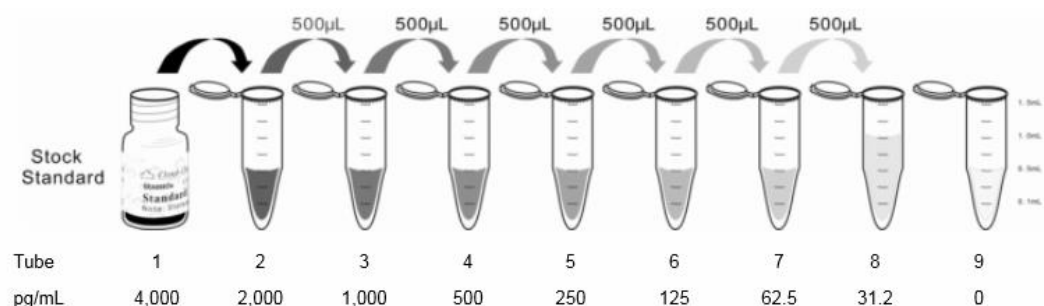
#### **2.3.1.1 Procalcitonin Reagent and Sample Preparation**

##### **2.3.1.1.1 Sample Dilution**

*Samples need to be brought to room temperature before being used for ELISA.*  
Both serum and sputum samples required no dilution for ELISA quantification.  
(Note: sputum processing produced a 1:3 sputum dilution)

### 2.3.1.1.2 Standard Preparation

Reconstitute the standard with 1.0mL Standard Diluent (room temperature) and mix gently. From the stock solution (4,000pg/mL) produce the following double dilution series:



**Figure 8 Standard double dilution series for USCN Life Sciences human PCT ELISA**

Standard diluent serves as the blank for the standard dilutions.

### 2.3.1.1.3 Reagent Preparation

*All reagents must be prepared fresh and utilised at room temperature.*

Assay Diluent A /Assay Diluent B Working Solution: Dilute 6mL of Assay Diluent A or B Concentrate (2x) with 6mL de-ionized water, to produce a total of 12mL.

Detection Reagent A/Detection Reagent B: Dilute to a 1:100 working concentration with Assay Diluent A/Assay Diluent B working solution.

### 2.3.1.2 Cathelicidin Reagent and Sample Preparation

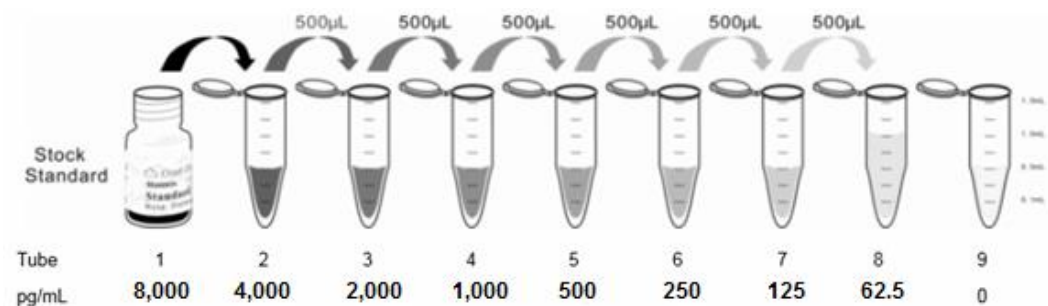
#### 2.3.1.2.1 Sample Dilution

*Samples need to be brought to room temperature before used for ELISA.*

Optimal dilution factor for ELISA assay was determined. 1:500 dilution for serum samples with 0.02mol/L PBS (pH=7-7.2). 1:5000 dilution for sputum samples with 0.02mol/L PBS (pH=7-7.2). (Note: sputum samples already diluted 1:3 so total dilution is 1:15000)

### 2.3.1.2.2 Standard Preparation

Reconstitute the standard with 1.0mL Standard Diluent (room temperature) and mix gently. From the stock solution (8,000pg/mL) produce the following double dilution series:



**Figure 9 Standard double dilution series for USCN Life Sciences human cathelicidin antimicrobial peptide ELISA**

Standard diluent serves as the blank for the standard dilutions.

### 2.3.1.2.3 Reagent Preparation

*All reagents must be prepared fresh and utilised at room temperature.*

Follow the procedure as above for PCT for making Detection Reagent A and B working solution.

### 2.3.1.3 $\beta$ -defensin 2 Reagent and Sample Preparation

#### 2.3.1.3.1 Sample Dilution

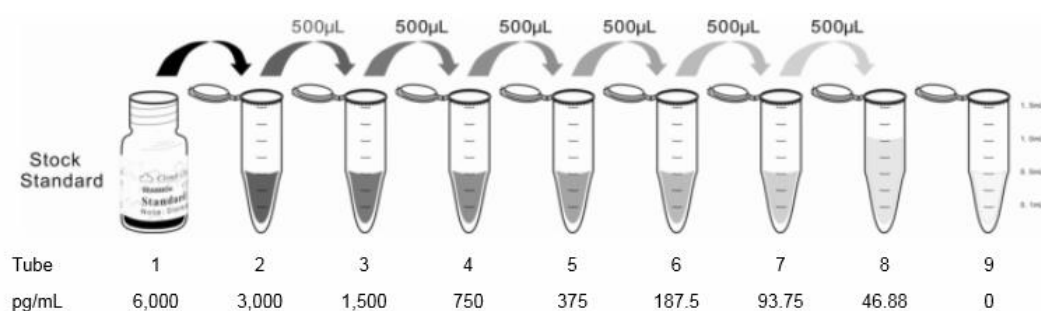
*Samples need to be brought to room temperature before used for ELISA.*

Both serum and sputum samples required no dilution for ELISA quantification.

(Note: sputum processing produced a 1:3 sputum dilution)

#### 2.3.1.3.2 Standard Preparation

Reconstitute the standard with 1.0ml Standard Diluent (room temperature) and mix gently. From the stock solution (6,000pg/mL) produce the following double dilution series:



**Figure 10 Standard double dilution series for USCN Life Sciences hBD-2 antimicrobial peptide ELISA**

Standard diluent serves as the blank for the standard dilutions.

### 2.3.1.3.3 Reagent Preparation

*All reagents must be prepared fresh and utilised at room temperature.*

Follow the procedure as above for PCT for making Detection Reagent A/Detection Reagent B working solution.

Quality Control: Reconstitute the vial with 150µL of standard diluent, mix gently and let stand for 10 minutes. Add 100µL to a well. Expected concentration is 540.1pg/mL. (Note: quality control was only provided for the newest kit)

### 2.3.1.4 General Assay Procedure for Procalcitonin, Cathelicidin and $\beta$ -defensin 2

1. Remove required numbers of strips from 96-well plate. Add 100µL of each standard dilution (7 wells), blank (1 well), quality control (if provided) and samples into predetermined wells. Cover with plate sealer and incubate for 2 hours at 37°C.
2. Aspirate wells, do not wash.
3. To each well add 100µL of Detection Reagent A working solution. Cover with plate sealer and incubate for 1 hour at 37°C.

4. Aspirate solution and wash three times using 350µL volume of Wash Buffer (1X PBS, 0.1% Tween 20) per well on automated plate washer (Thermofisher Scientific™ Wellwash Versa™ Microplate Washer).
5. To each well add 100µL of Detection Reagent B working solution. Cover with plate sealer and incubate for 30 minutes at 37°C.
6. Repeat wash step but for a total of five times.
7. Add 90µL of TMB substrate solution to each well. Use new plate sealer and incubate for 15-25 minutes. Wells should turn blue.
8. After incubation add 50µL of Stop solution and tap plate gently on side to mix. Liquid should turn yellow.
9. Immediately measure optical density (OD) at 450nm using microplate reader (Thermofisher Scientific™ Multiskan Go™ Microplate Spectrophotometer).

### **2.3.2 Hycult Biotech Human LL-37 ELISA Kit**

This commercially available kit is a solid-phase enzyme-linked immunosorbent assay based on the sandwich principle for the determination of human LL-37. Samples are incubated in wells that are coated with antibodies specific for human LL-37. The added biotinylated tracer antibody will bind to the captured LL-37. The Streptavidin-peroxidase conjugate will bind to the tracer antibody. The S-P conjugate will react with the tetramethylbenzidine (TMB) substrate to produce a blue colour. The enzymatic action is then stopped by the addition of oxalic acid to produce a yellow colour. LL-37 is the antibacterial C-terminus (37 amino acids) of hCAP 18. ELISA kit was run according to manufacturer's protocol.

#### **2.3.2.1 Sample Dilution**

All serum samples required a 1:20 dilution with supplied wash/dilution buffer. Sputum samples required a 1:200 dilution with supplied wash/dilution buffer.

### **2.3.2.2 Reagent Preparation**

*All reagents are to be brought to room temperature prior to use. All required reagents are to be prepared fresh.*

#### Wash/Dilution Buffer:

1. Determined the required volume for the amount of dilutions being done.
2. Mix 1 part 20X concentrated wash/dilution buffer A with 9 parts de-ionized water.
3. Mix 1 part 40X concentrated wash/dilution buffer B with 19 parts de-ionized water.
4. Combine both solutions equally and mix well.

#### Tracer Solution:

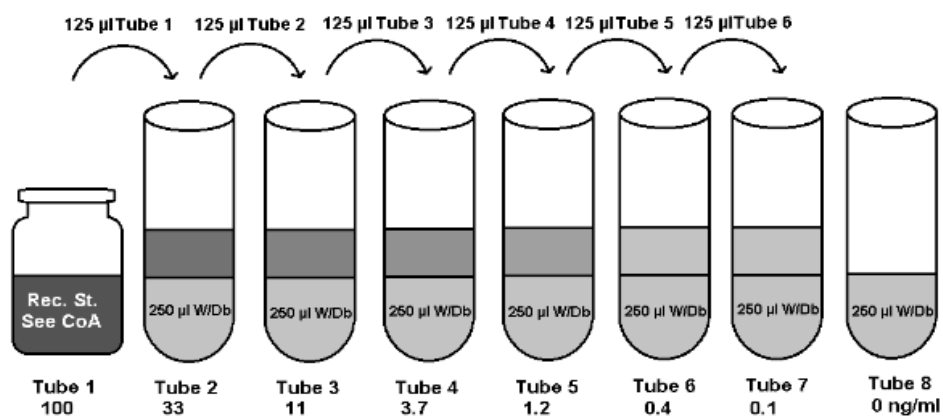
1. Reconstitute the tracer with 1mL of de-ionized water.
2. Determine required tracer volume for number of test wells.
3. Dilution requires 1 part of reconstituted tracer and 11 parts of wash/dilution buffer. Mix thoroughly.

#### Streptavidin-peroxidase Solution:

1. Reconstitute the streptavidin-peroxidase with 1mL of de-ionized water.
2. Determine required streptavidin-peroxidase volume for number of test wells.
3. Dilution requires 1 part of reconstituted streptavidin-peroxidase with 23 parts of wash/dilution buffer. Mix thoroughly.

### **2.3.2.3 Standard Preparation**

Reconstitute the standard with 0.5mL de-ionized water. Prepare the standard serial dilution using wash/dilution buffer from the stock standard (242ng/mL) as follows:



CoA: Certificate of Analysis, Rec. St: Reconstituted Standard, W/Db: Wash/Dilution buffer

**Figure 11 Standard dilution series for Hycult LL-37 ELISA**

From the Certificate of Analysis (CoA): the amount of wash/dilution buffer to be used in tube 1 is 0.213mL.

### 2.3.2.4 ELISA Protocol

*All reagents are to be brought to room temperature prior to use. It is important that the same pipetting order is followed.*

1. Remove required number of microwell strips.
2. In duplicate add 100µL of standard, sample, or controls to predetermined wells.
3. Cover with plate sealer and incubate for 1 hour at room temperature.
4. Wash plate four times using automated plate washer (Thermofisher Scientific™ Wellwash Versa™ Microplate Washer) (300ul/well) with Wash Buffer (1x PBS, 0.1% Tween 20).
5. Add 100µL of diluted tracer to each well, cover and incubate for 1 hour at room temperature.
6. Repeat wash procedure (Step 4).
7. Add 100µL of diluted streptavidin-peroxidase to each well, cover and incubate for one hour at room temperature.



8. Repeat wash procedure (Step 4).
9. Add 100 $\mu$ L of TMB substrate to each well, cover tray with tin foil (light sensitive) and incubate for 30 minutes at room temperature.
10. Add 100 $\mu$ L of stop solution. Gently swirl the plate to mix and to remove any air bubbles.
11. Within 30 minutes read the plate using the microplate reader (Thermofisher Scientific<sup>TM</sup> Multiskan Go<sup>TM</sup> Microplate Spectrophotometer) at 450nm.

### **2.3.3 Vitamin D Cusabio ELISA Kits**

Two separate competitive inhibition enzyme immunoassay kits for the quantification of 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations in human serum. The plates come pre-coated with the specific antibody. Samples are added to the wells with Horseradish Peroxidase (HRP) conjugated 25(OH)D<sub>3</sub> (or 1,25(OH)<sub>2</sub>D<sub>3</sub>) Hapten. There is then a competitive inhibition reaction between HRP-conjugated 25(OH)D<sub>3</sub> (or 1,25(OH)<sub>2</sub>D<sub>3</sub>) Hapten and the 25(OH)D<sub>3</sub> (or 1,25(OH)<sub>2</sub>D<sub>3</sub>) present in the samples. Substrate solution is then added and the colour reaction develops inversely to the actual amount of 25(OH)D<sub>3</sub> (or 1,25(OH)<sub>2</sub>D<sub>3</sub>) present in the sample. This colour reaction is then stopped and absorbency read. Concentrations determined by producing a standard curve using four-parameter logistic curve fit. ELISA kits were run according to manufacturer's protocol.

#### **2.3.3.1 Human 25-hydroxyvitamin D<sub>3</sub> Reagent and Sample Preparation**

##### **2.3.3.1.1 Sample Dilution**

The optimal dilution for serum samples was 1:30, diluted using the provided Sample Diluent. No dilution is required for the sputum dilution. (Note: sputum is already diluted 1:3 due to the sputum processing procedure)

### 2.3.3.1.2 Standard Preparation

Reconstitute the vial with 0.5ml de-ionized water, mix thoroughly and let sit for 15 minutes. Dilute the stock solution (4,000µg/L) 40-fold to produce Standard 4 (100µg/L). Then dilute Standard 4 as follows to produce the required standard dilutions:

**Table 2 Required standard dilution series for 25(OH)D<sub>3</sub> ELISA**

Tube	S4	S3	S2	S1	S0
Conc. (µg/L)	100	80	40	20	0
S4 (µL)	200	160	80	40	0
Samples Diluent (µL)	0	40	120	160	200

### 2.3.3.1.3 Reagent Preparation

High Value Control (HVC): Reconstitute with 0.5mL of de-ionized water. Mix thoroughly and then allow it to sit for 15 minutes. Dilute reconstituted high value control 1:40 with standard diluent.

Middle Value Control (MVC): Reconstitute with 0.5mL of de-ionized water. Mix thoroughly and then allow it to sit for 15 minutes. Dilute reconstituted middle value control 1:40 with standard diluent.

### 2.3.3.2 Human 1,25-dihydroxyvitamin D<sub>3</sub> Reagent and Sample Preparation

#### 2.3.3.2.1 Sample Dilution

The optimal dilution for serum samples was 1:10, diluted using the provided Sample Diluent. No dilution is required for the sputum dilution. (Note: sputum is already diluted 1:3 due to the sputum processing procedure)

#### 2.3.3.2.2 Standard Preparation

Reconstitute the vial with 0.5mL de-ionized water, mix thoroughly and let sit for 15 minutes. Dilute the stock solution (200,000fmol/L) 40-fold to produce

Standard 4 (5000 fmol/L). Then dilute standard 4 as follows to produce required standard dilutions:

**Table 3 Required standard dilution series for 1,25(OH)<sub>2</sub>D<sub>3</sub> ELISA**

Tube	S4	S3	S2	S1	S0
Concentration (fmol/L)	5000	4000	2000	1000	0
S4 (μL)	0	160	80	40	0
Sample Diluent (μL)	0	40	120	160	200

### 2.3.3.2.3 Reagent Preparation

High Value Control (HVC): Reconstitute with 0.5mL of de-ionized water. Mix thoroughly and then allow it to sit for 15 minutes. Dilute reconstituted high value control 1:40 with standard diluent.

Middle Value Control (MVC): Reconstitute with 0.5mL of de-ionized water. Mix thoroughly and then allow it to sit for 15 minutes. Dilute reconstituted middle value control 1:40 with standard diluent.

### 2.3.3.3 General Assay Procedure for 25-hydroxyvitamin D<sub>3</sub> and 1,25-dihydroxyvitamin D<sub>3</sub>

1. Remove the required number of well strips.
2. Set one blank well with no solution.
3. Add 50μL of standard, control and diluted sample to pre-determined well. Then add 50μL of HRP-conjugate immediately to each well. Mix gently by shaking plate for one minute.
4. Cover with plate sealer and incubate for one hour at 37°C.
5. Aspirate and wash each well four times with 300μL of Wash Buffer (1X PBS, 0.1% Tween 20) using an autowasher (Thermofisher Scientific™ Wellwash Versa™ Microplate Washer).

6. Add 50 $\mu$ L of substrate A and 50 $\mu$ L of Substrate B to each well. Then incubate for 15 minutes at 37°C. Protect from light.
7. At end of incubation add 50 $\mu$ L of Stop Solution to each well and tap plate gently to mix.
8. Within five minutes read the optical density (OD) using a microplate reader (Thermofisher Scientific<sup>TM</sup> Multiskan Go<sup>TM</sup> Microplate Spectrophotometer) set to 450nm.

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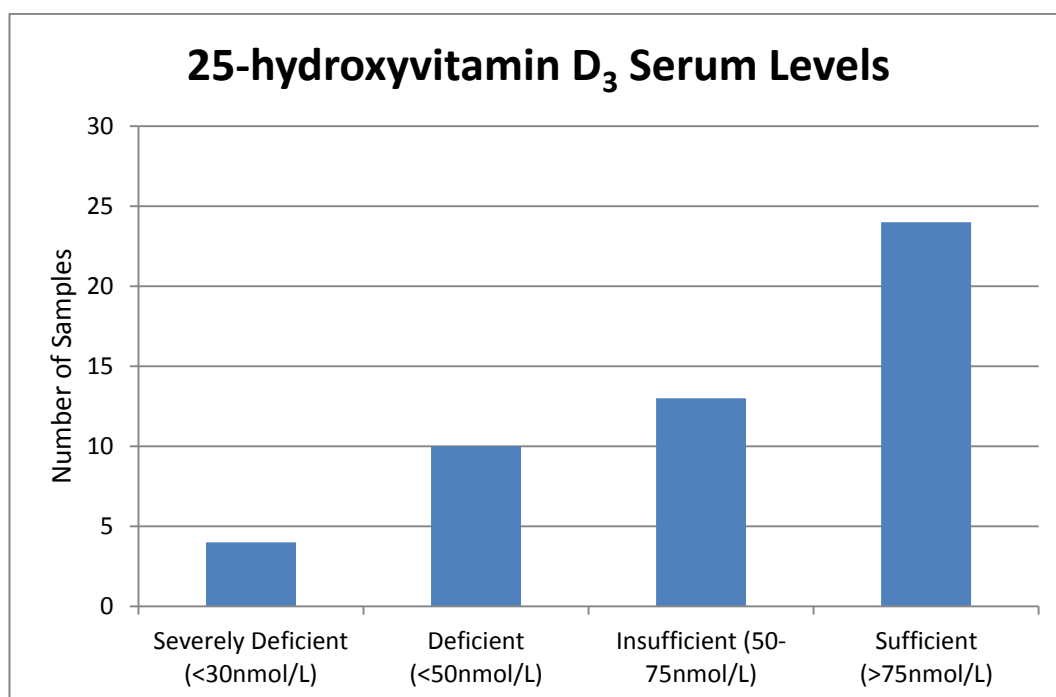
# Chapter Three: Results

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## 3.1 ELISA Results

### 3.1.1 25-hydroxyvitamin D<sub>3</sub> Concentrations

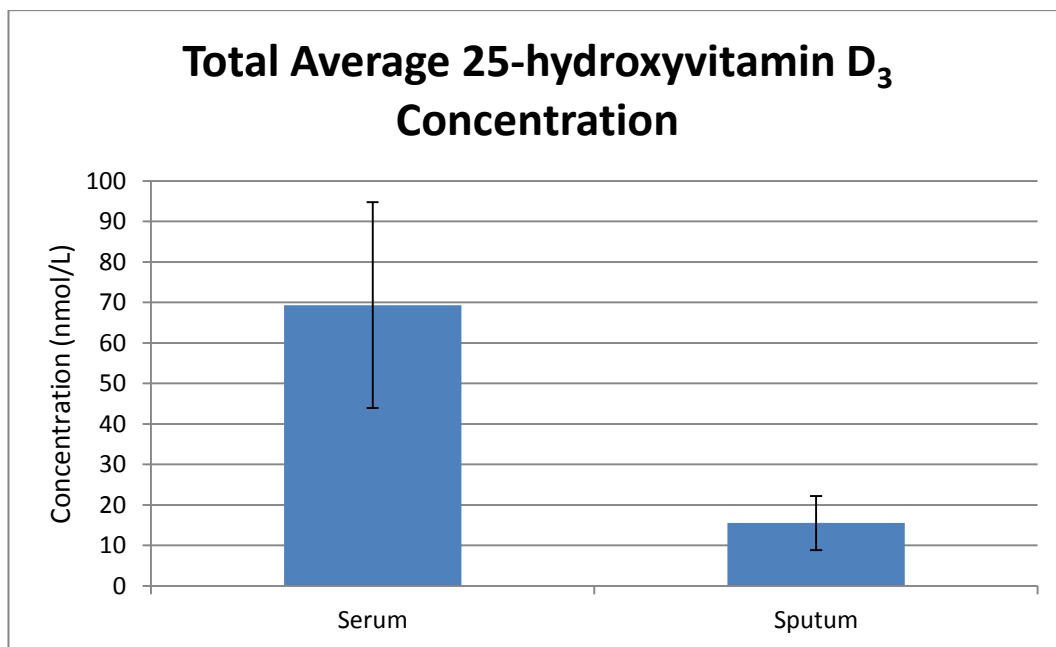
The mean serum 25(OH)D<sub>3</sub> level was 69.34nmol/L (range=19.21-129.59nmol/L, n=51) and median 71.31nmol/L for all samples across the 54 week period. A total of 14 out of 51 samples (27.45%) had deficient serum 25(OH)D levels (<50nmol/L), of which only four samples (7.84%) were severely deficient (<30nmol/L). Insufficient 25(OH)D levels (50-75nmol/L) were identified in 13 out of 51 samples (25.49%). A total of 24 (47.06%) of the samples had sufficient 25(OH)D levels >75nmol/L. Therefore 52.9% of all samples had suboptimal vitamin D levels.



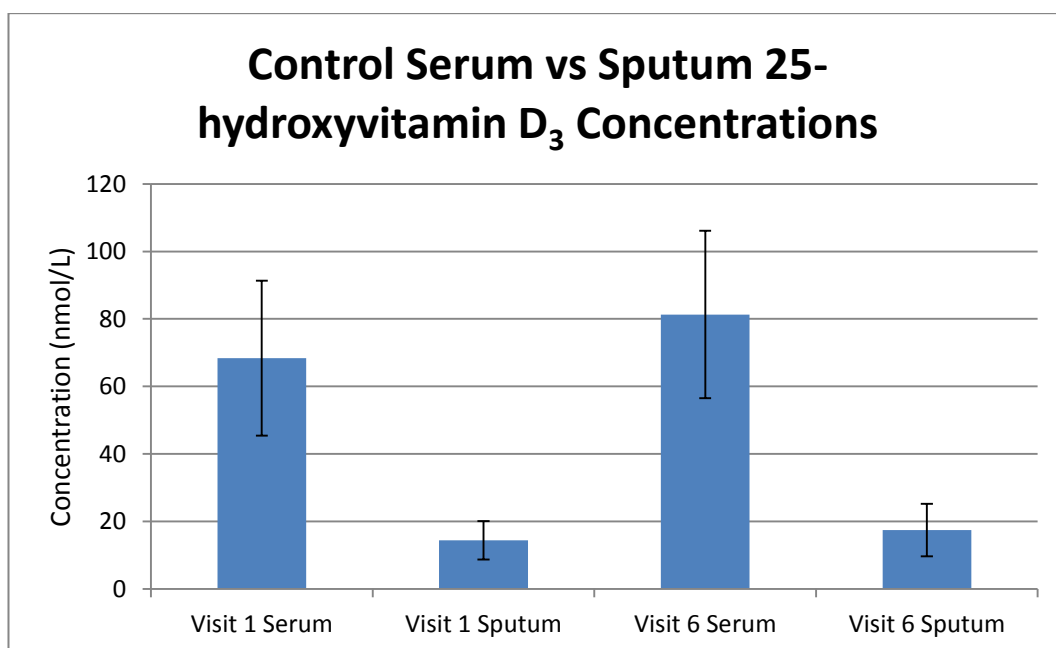
**Figure 12 Graph showing the breakdown of bronchiectasis serum samples with their 25(OH)D cut-off points**

The mean sputum 25(OH)D<sub>3</sub> level was 15.53nmol/L (range=4.83-31.88nmol/L, n=50) and the median 14.40nmol/L. There was a significant difference (p=0.0000)

between the total mean serum and sputum 25(OH)D<sub>3</sub> concentrations (Figure 13). The Paired T-test further supported the significant difference between serum and sputum. Using just the control samples there was a significance found between visit one serum and sputum (p=0.0000001), and between visit six serum and sputum (p=0.0001) (Figure 14).

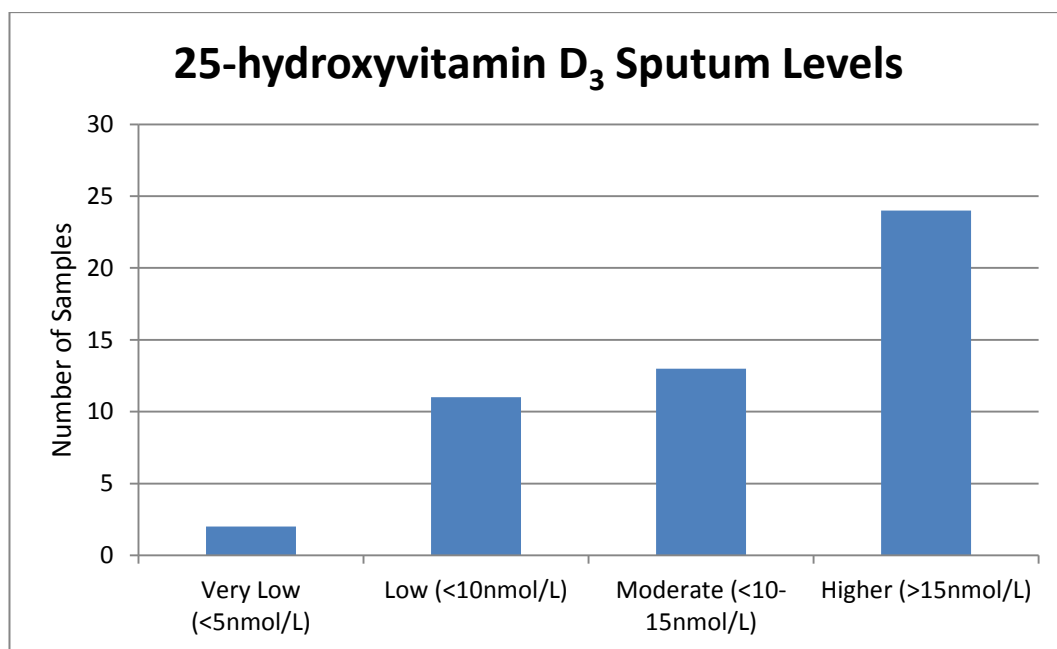


**Figure 13** Graph showing the overall serum and sputum 25(OH)D<sub>3</sub> concentrations for all possible samples



**Figure 14** Graph showing control serum vs. sputum 25(OH)D<sub>3</sub> concentrations

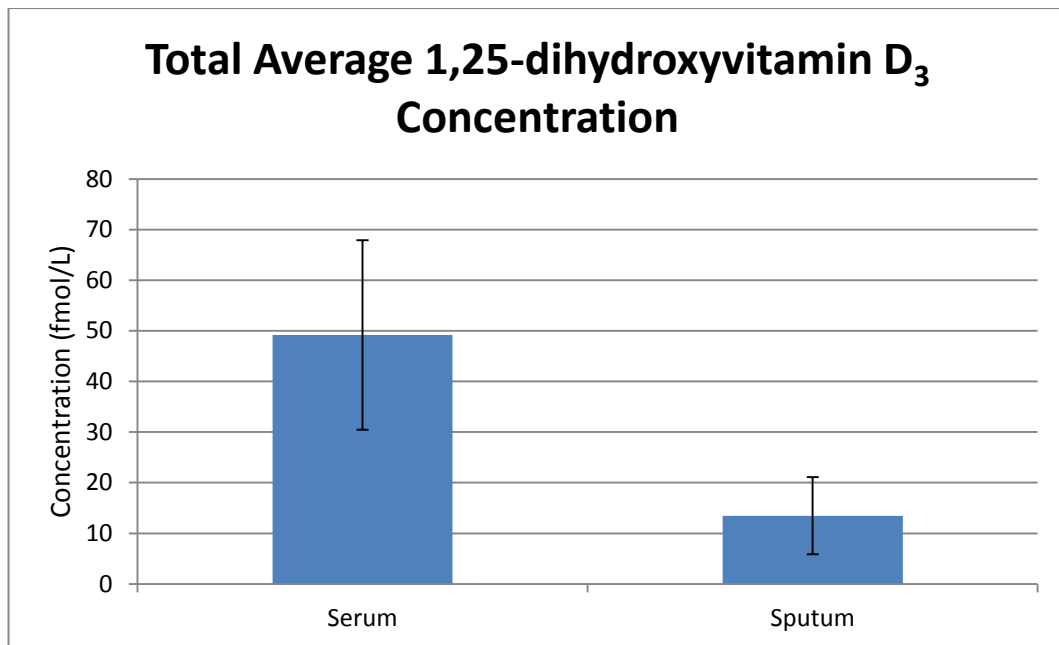
The 25(OH)D<sub>3</sub> sputum levels (Figure 15) followed a similar trend to the 25(OH)D<sub>3</sub> serum levels (Figure 12). Levels were estimated and only used for providing a comparison; they are not true consensus levels.



**Figure 15** Graph showing the breakdown of bronchiectasis sputum samples with their estimated 25(OH)D cut-off points

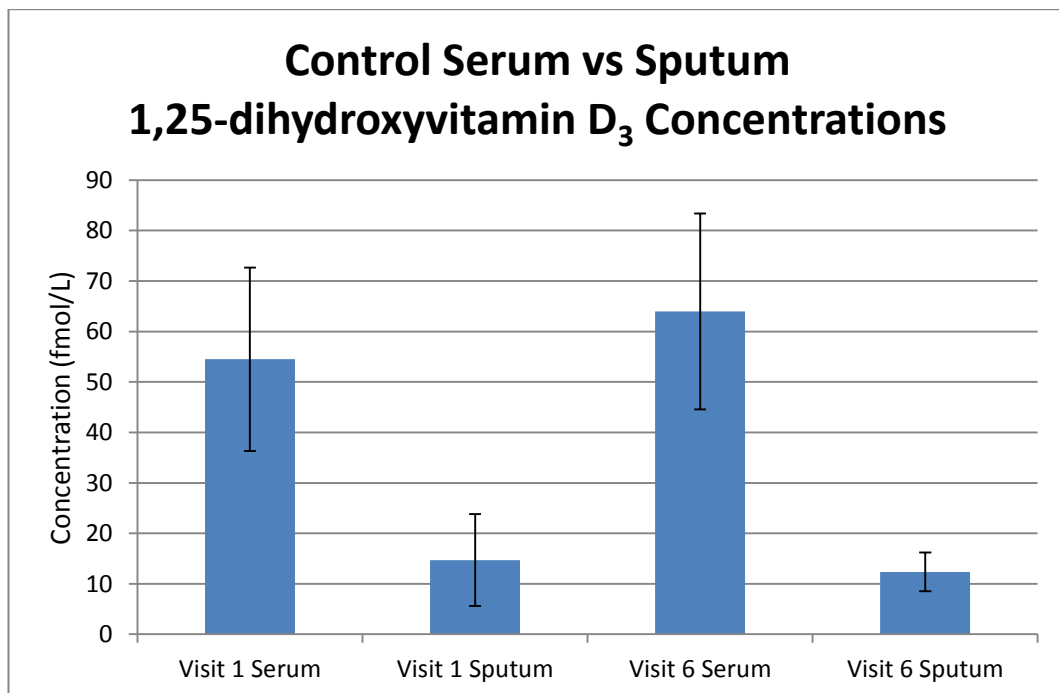
### 3.1.2 1,25-dihydroxyvitamin D Concentrations

The overall mean serum concentration for 1,25(OH)<sub>2</sub>D<sub>3</sub> over a period of 54 weeks is 49.18pmol/L (range=13.17-94.57pmol/L, n=54) and the median 48.08pmol/L . The overall mean sputum 1,25(OH)<sub>2</sub>D<sub>3</sub> concentration over a period of 54 weeks is 13.48pmol/L (range=0.82-42.96, n=53) and the median 12.25pmol/L. The mean values for serum and sputum can be seen in Figure 16 and are statistically significant (p=0.0000).



**Figure 16** Graph showing the overall serum and sputum 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations for all possible samples

The use of a Paired T-Test further confirmed that the 1,25(OH)<sub>2</sub>D sputum levels are significantly lower than in serum. This was achieved by analysing only the serum and sputum baseline periods (Visit 1 & 6) (Figure 17). The significance between visit one serum and sputum was  $p=0.00000064$  and between visit six serum and sputum was  $p=0.000007$ .



**Figure 17** Graph showing control serum vs sputum 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations



### **3.1.3 25-hydroxyvitamin D<sub>3</sub> vs 1,25-dihydroxyvitamin D<sub>3</sub> Association**

There was a correlation found between serum 25(OH)D<sub>3</sub> levels and serum 1,25(OH)<sub>2</sub>D<sub>3</sub> levels ( $\rho=0.6910$ ,  $p=0.00000008$ ) using Spearman's non-parametric correlations. This indicates the presence of a strong positive correlation between serum 25(OH)D<sub>3</sub> and serum 1,25(OH)<sub>2</sub>D<sub>3</sub> levels. The p-value was determined using a Paired T-Test, using only paired samples from all visits (total of 47 paired samples). The mean serum level of these paired samples was 68.24 +/- 25.84nmol/L for 25(OH)D<sub>3</sub> and 49.84 +/- 19.35pmol/L for 1,25(OH)<sub>2</sub>D<sub>3</sub>.

Spearman's non-parametric correlation revealed no correlation between serum 25(OH)D levels and sputum 1,25(OH)<sub>2</sub>D levels ( $\rho=-0.1560$ ). But 1,25(OH)<sub>2</sub>D sputum levels are significantly lower than 25(OH)D serum levels ( $p=0.0000$ ). The p-value was determined using a Paired T-Test using all paired samples from all visits, using a total of 45 paired samples. The mean level of these paired samples was 68.65 +/- 25.62nmol/L for 25(OH)D serum and 13.54 +/- 7.37pmol/L for sputum 1,25(OH)<sub>2</sub>D.

### **3.1.4 Cathelicidin Concentrations**

Two separate commercially available ELISA kits were used in the determination of cathelicidin concentrations. When the data produced by the two separate kits are compared the results do not correlate. This may be due to the two ELISA kits measuring different components of parts of the active LL-37 or cathelicidin.

#### **3.1.4.1 Correlation between Hycult LL-37 vs USCNK CAMP Levels**

Performed a Spearman's Rank Correlation between these two kits serum levels and expected that the levels should at least relate to each other. A total of 23 sample pairs were used in the correlation. A very weak negative correlation was found ( $\rho=-0.1413$ ) between LL-37 serum levels and CAMP serum levels.

### 3.1.4.2 Hycult LL-37 ELISA Concentration

The mean serum cathelicidin concentration was 57.54ng/mL (range=23.32-127.36ng/mL, n=38) and a median of 50.44ng/mL. Only two sputum samples (duplicates) were run on this plate. One sputum sample was 1971.26ng/mL (respective serum value=49.96ng/mL) and the other sputum sample was 2396.59ng/mL (respective serum value=33.13ng/mL). This produces a difference of 39-fold and 72-fold respectively, between serum and sputum levels for these two samples.

### 3.1.4.3 USCNK CAMP ELISA Concentration

The mean serum CAMP concentration was 569.12ng/mL (range=89.41-1760.61ng/mL, n=32) and median of 412.67ng/mL. The mean sputum CAMP concentration was 60,443.29ng/mL (or 60.44µg/mL) (range=605.96-196545.71ng/mL). The median CAMP sputum level was 55,728.44ng/mL (or 55.73ug/mL). The difference between these two means was statistically significant ( $p=0.00000001$ ) (Figure 18). This indicates an average 106-fold increase in sputum levels, compared to serum levels. Limited paired samples were available to carry out Paired T-Test. No significant difference ( $p=0.125$ ) was found in CAMP serum levels across visits 1-5.

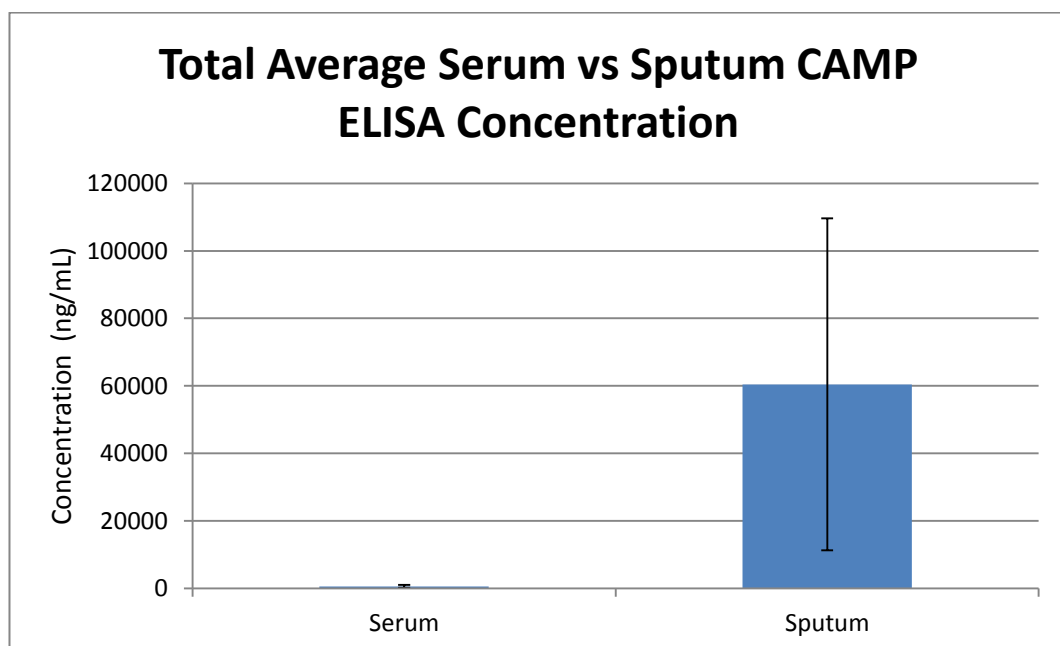
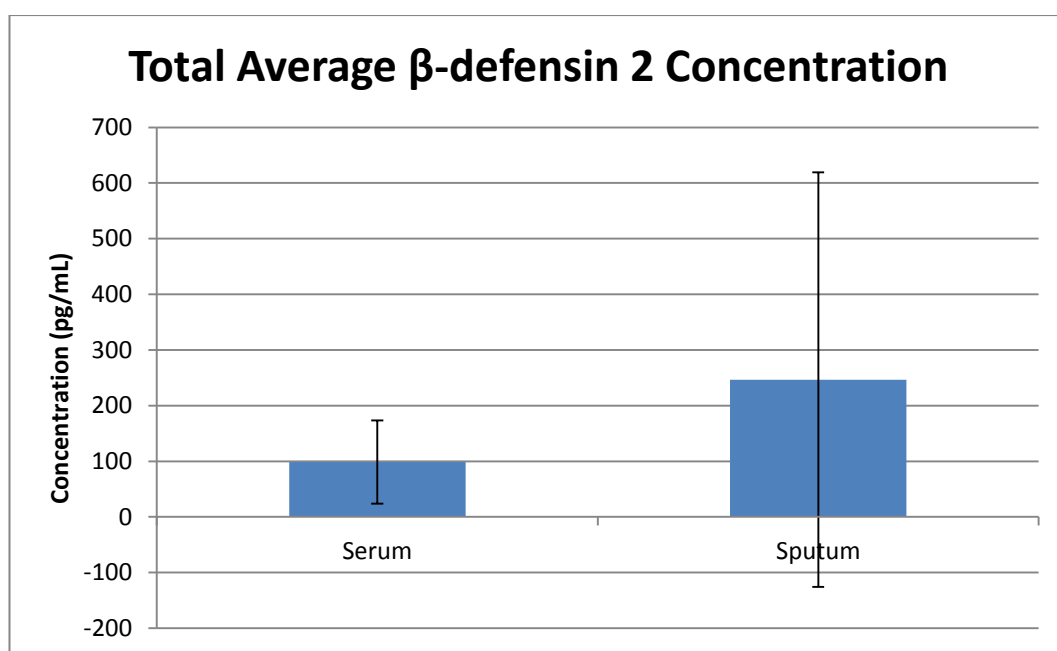


Figure 18 Total average serum vs sputum CAMP concentration for all possible samples

### 3.1.5 $\beta$ -defensin 2 Concentrations

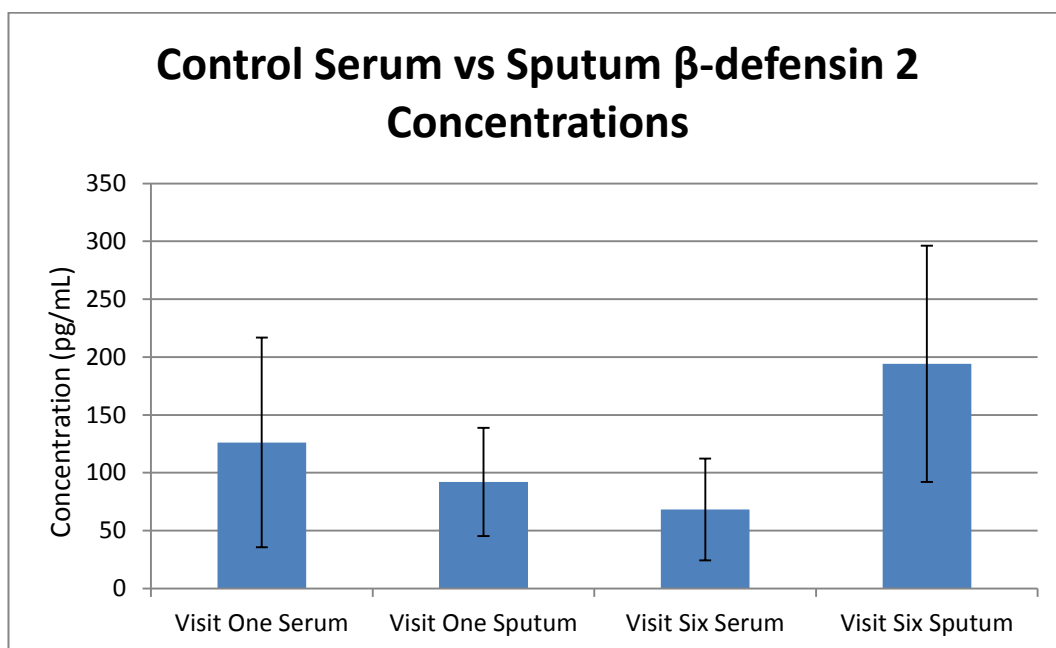
From all possible samples the total average concentration for hBD-2 was estimated. The total average serum concentration across the 54 week period is 98.68pg/mL (range=8.05-325pg/mL, n=56) and median 72.88pg/mL. The average total sputum concentration is 165.79pg/mL (range=30.07-574.20pg/mL, n=52) and median 129.76pg/mL. The mean was 246.71pg/mL (range=30.07-2121.03 , n=55) and median 136.84pg/mL if the outlier has been included. The outlier individual has very high hBD-2 sputum concentrations at visit five (1090.66pg/mL), visit six (2121.03pg/mL) and visit nine (1493.29pg/mL) that were skewing the other remaining data. This data reveals a significant difference between serum and sputum hBD-2 concentrations ( $p=0.0044$ ) (including outlier) and still significant if outlier removed ( $p=0.0026$ ).



**Figure 19** Graph of the total average hBD- 2 serum and sputum concentrations (outlier inclusive)

Only the baseline levels (Visit 1 & 6) were used for determining if there is any significant difference between the serum and sputum hBD-2 concentrations. P-values were determined using a Paired T-Test. There was no significance between visit one serum and sputum ( $p=0.23$ , n=14), or visit six serum and sputum ( $p=0.12$ ,

n=11). If the outlier was removed from calculations there was a significant difference found between visit six serum and sputum concentrations ( $p=0.006976$ ,  $n=10$ ) (Figure 20).



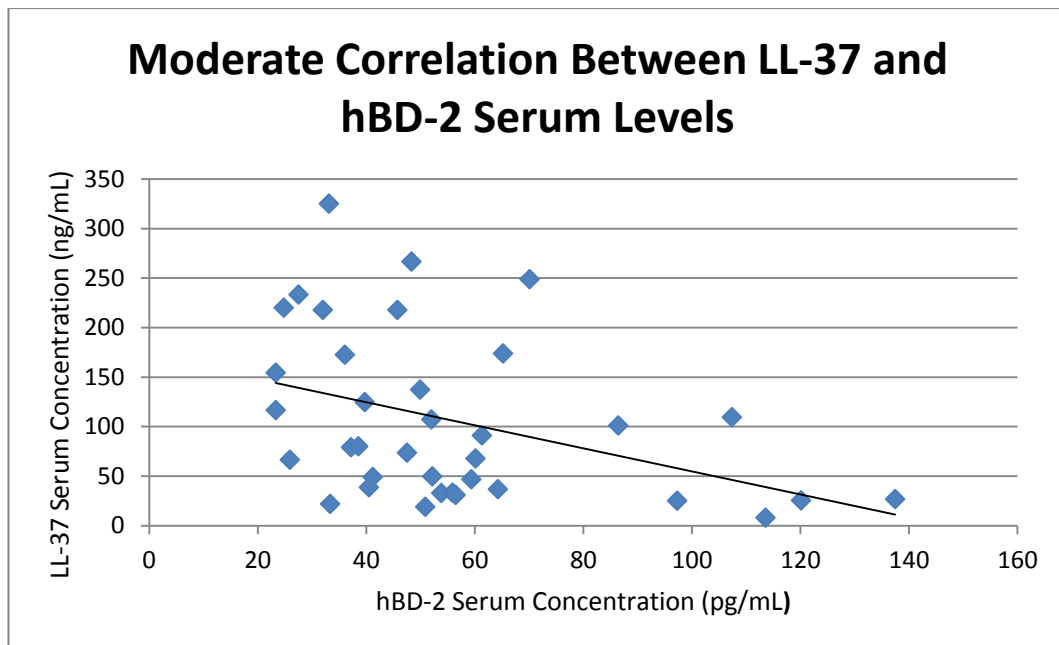
**Figure 20** Graph of control serum vs sputum hBD-2 concentrations (outlier removed)

### 3.1.6 Correlation Between Cathelicidin and $\beta$ -defensin 2

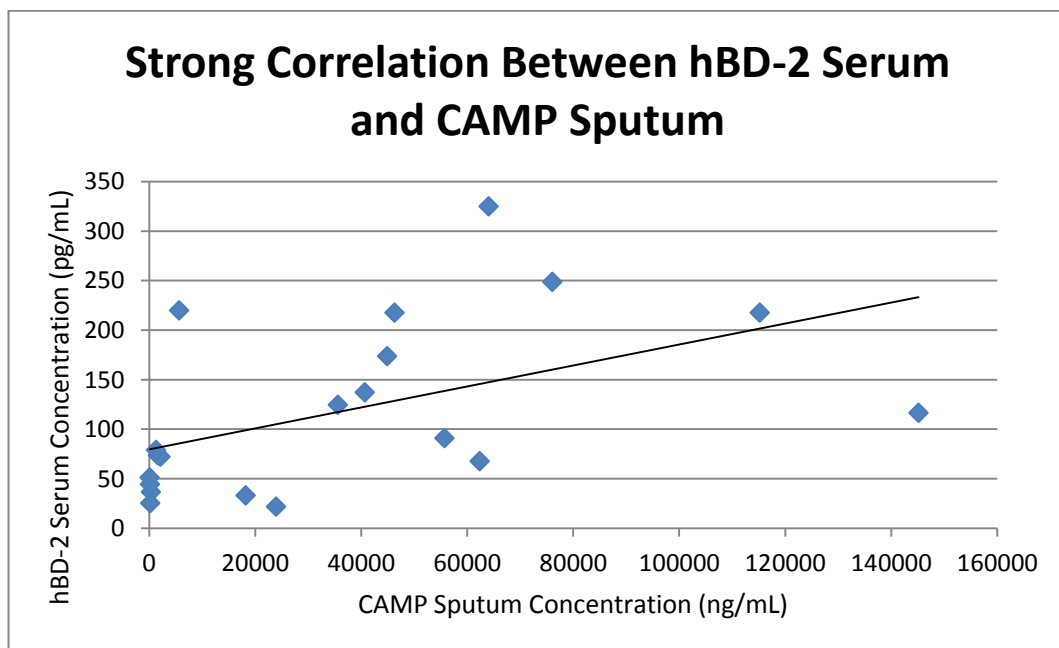
Spearman's non-parametric correlation coefficient was calculated to determine if any correlations exist between cathelicidin and hBD-2 concentrations. Both Hycult LL-37 and USCNK CAMP concentrations were used for analysis. A strong positive correlation ( $\rho=0.633083$ ) was found between hBD-2 serum and CAMP sputum levels (Figure 22). A moderate negative correlation ( $\rho=-0.44273$ ) was found between LL-37 serum and hBD-2 serum level (Figure 21).

**Table 4** Spearman correlation coefficient (number of pairs) for hBD-2 and cathelicidin

	LL-37 Serum	CAMP Serum	CAMP Sputum
hBD-2 Serum	<b>-0.44273 (36)</b>	0.05495 (29)	<b>0.633083 (20)</b>
hBD-2 Sputum	-0.19132 (35)	0.00462 (25)	0.120301 (20)



**Figure 21** A moderate negative correlation exists between LL-37 and hBD-2 concentrations



**Figure 22** A strong positive correlation exists between hBD-2 serum and CAMP sputum concentrations

### 3.1.7 Association of Vitamin D and Antimicrobial Peptides

#### 3.1.7.1.1 LL-37 Association with Vitamin D Metabolites

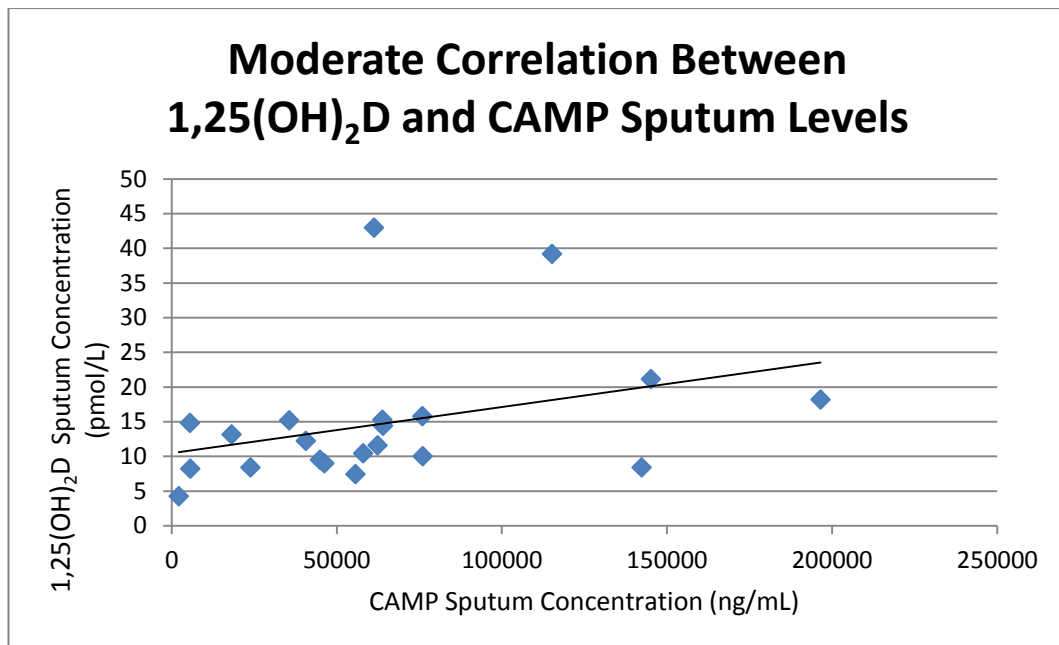
Spearman rank correlation has revealed a very weak correlation between serum LL-37 levels and serum 25(OH)D levels ( $\rho=-0.04813$ ) or sputum 25(OH)D levels ( $\rho=0.154786$ ). No associations have been found with either 1,25(OH)<sub>2</sub>D serum ( $\rho=-0.20244$ ) or 1,25(OH)<sub>2</sub>D sputum levels ( $\rho=0.160275$ ).

#### 3.1.7.1.2 CAMP Association with Vitamin D Metabolites

The association between CAMP concentrations and the measured vitamin D metabolites was assessed using Spearman's Rank Correlation. This revealed only weak associations between serum and sputum CAMP and vitamin D (Table 5). A moderate positive correlation ( $\rho=0.497403$ ) was found between CAMP sputum levels and 1,25(OH)<sub>2</sub>D sputum levels (Figure 23).

**Table 5 Spearman correlation coefficient (number of pairs) for CAMP and vitamin D metabolites**

	CAMP Serum	CAMP Sputum
25(OH)D Serum	-0.3287 (24)	-0.015038 (20)
25(OH)D Sputum	0.193846 (26)	0.323377 (21)
1,25(OH) <sub>2</sub> D Serum	-0.30037 (26)	0.062308 (25)
1,25(OH) <sub>2</sub> D Sputum	-0.03297 (27)	<b>0.497403 (20)</b>



**Figure 23 A moderate positive correlation exists between 1,25(OH)<sub>2</sub>D and CAMP sputum concentrations**

### 3.1.7.2 β-defensin 2 Association with Vitamin D Metabolites

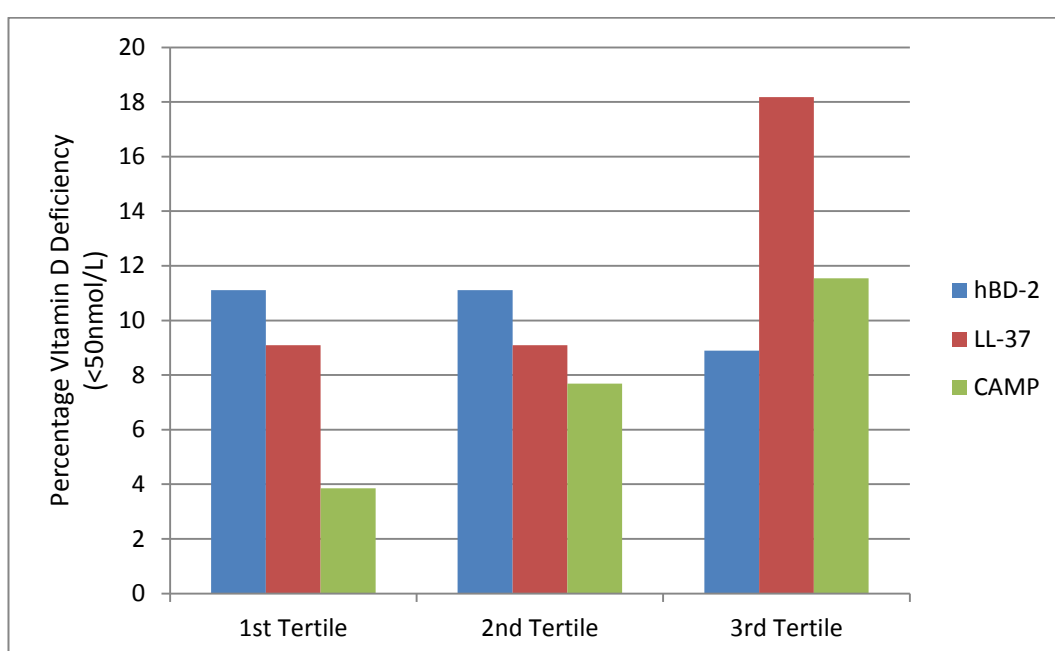
Spearman's rank correlation has revealed that only weak correlations exist between any of the measured vitamin D metabolites concentrations and hBD-2 concentrations. All rho values indicate a very weak correlation (Table 6).

**Table 6 Spearman correlation coefficient (number of pairs) for hBD-2 and vitamin D metabolites**

	hBD-2 Serum	hBD-2 Sputum
25(OH)D Serum	0.051252	0.063425
25(OH)D Sputum	-0.1215	0.044475
1,25(OH) <sub>2</sub> D Serum	-0.00734	-0.09424
1,25(OH) <sub>2</sub> D Sputum	-0.01284	0.026334

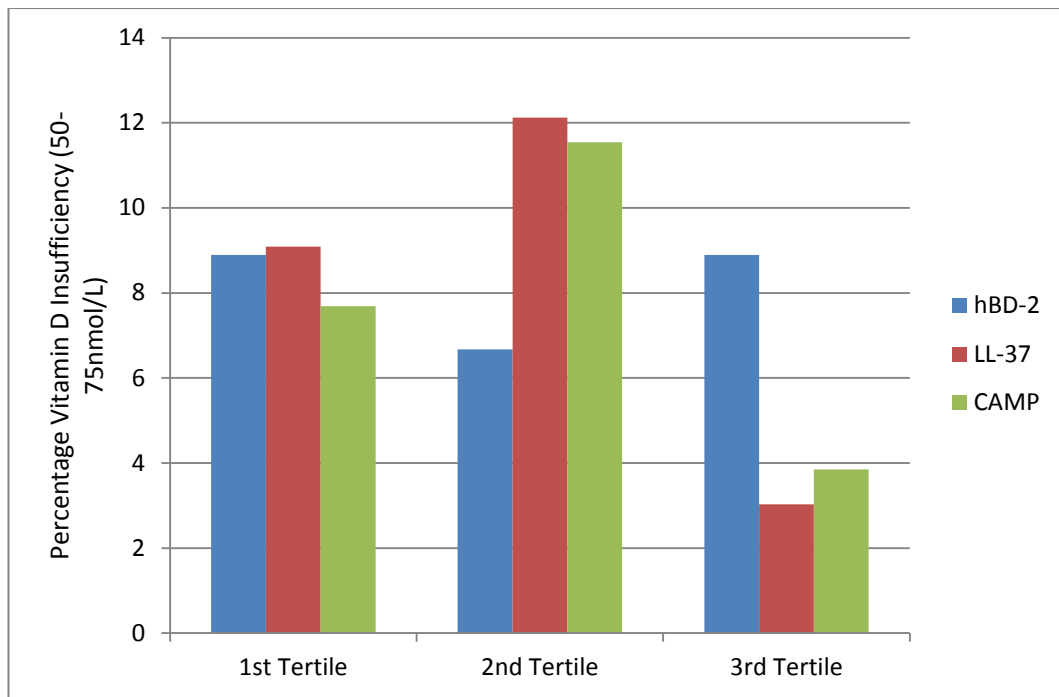
### 3.1.7.3 Tertiles

The data underwent a log transformation to enable further statistical analysis to be done. The data now adopts a normal distribution meaning that parametric tests can now be used. Tertiles are required for grouping 1,25(OH)<sub>2</sub>D<sub>3</sub>, LL-37, CAMP and hBD-2 which have no standardised concentrations. These AMP tertile groups were then compared to their samples corresponding vitamin D status (serum 25(OH)D<sub>3</sub> concentration) (VDD-Figure 24, VD insufficiency-Figure 25, VD sufficiency-Figure 26).

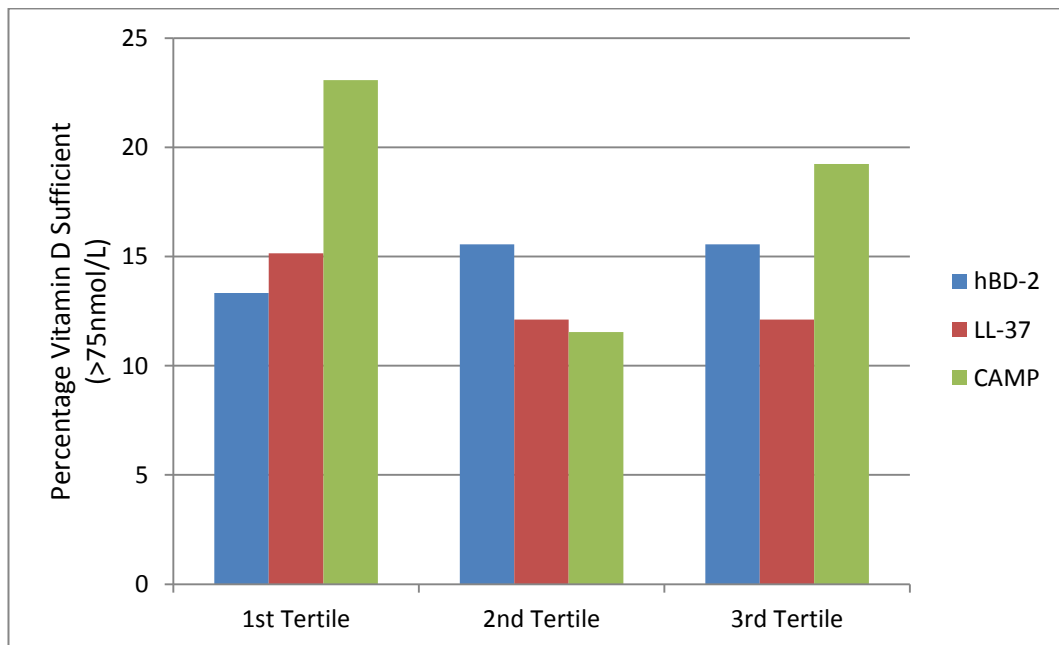


**Figure 24 Relationship between percentage of vitamin D deficiency (<50nmol/L) and the concentrations of hBD-2, LL-37 and CAMP**





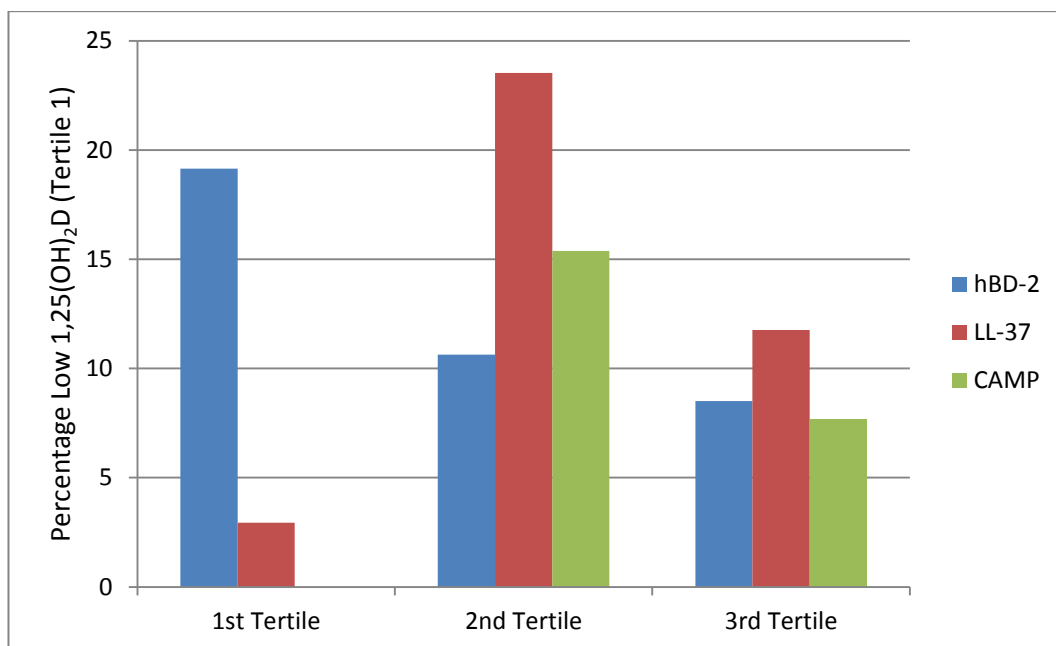
**Figure 25 Relationship between percentage of vitamin D insufficiency (50-75nmol/L) and the concentrations of hBD-2, LL-37 and CAMP**



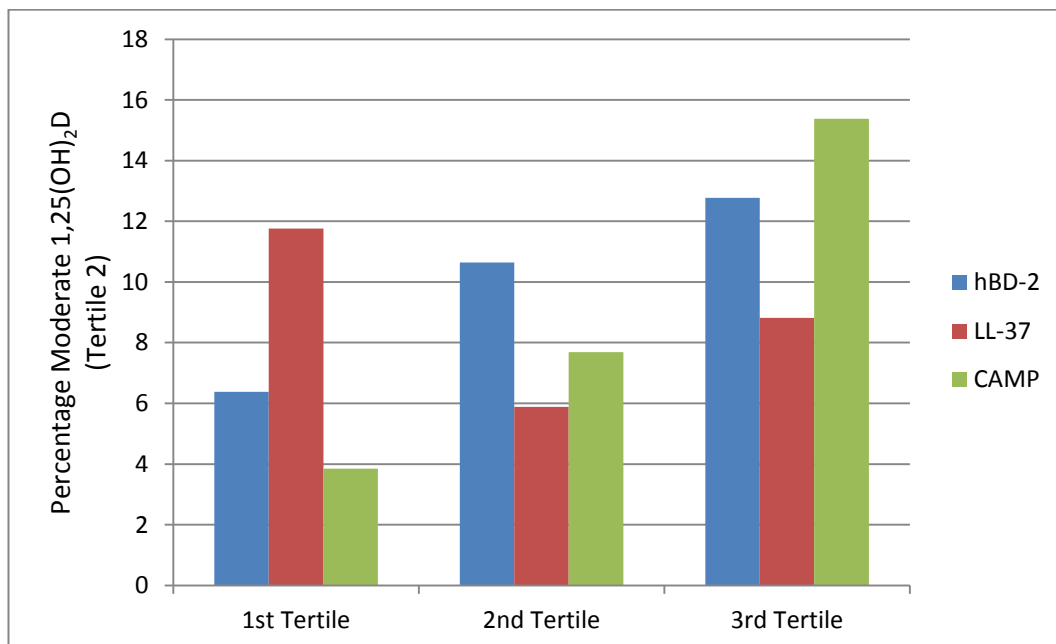
**Figure 26 Relationship between percentage of vitamin D sufficiency (>75nmol/L) and the serum concentrations of hBD-2, LL-37 and CAMP**

Due to no consensus levels for available for 1,25(OH)<sub>2</sub>D, the data was divided into low, moderate and high using tertiles. The serum 1,25(OH)<sub>2</sub>D concentrations

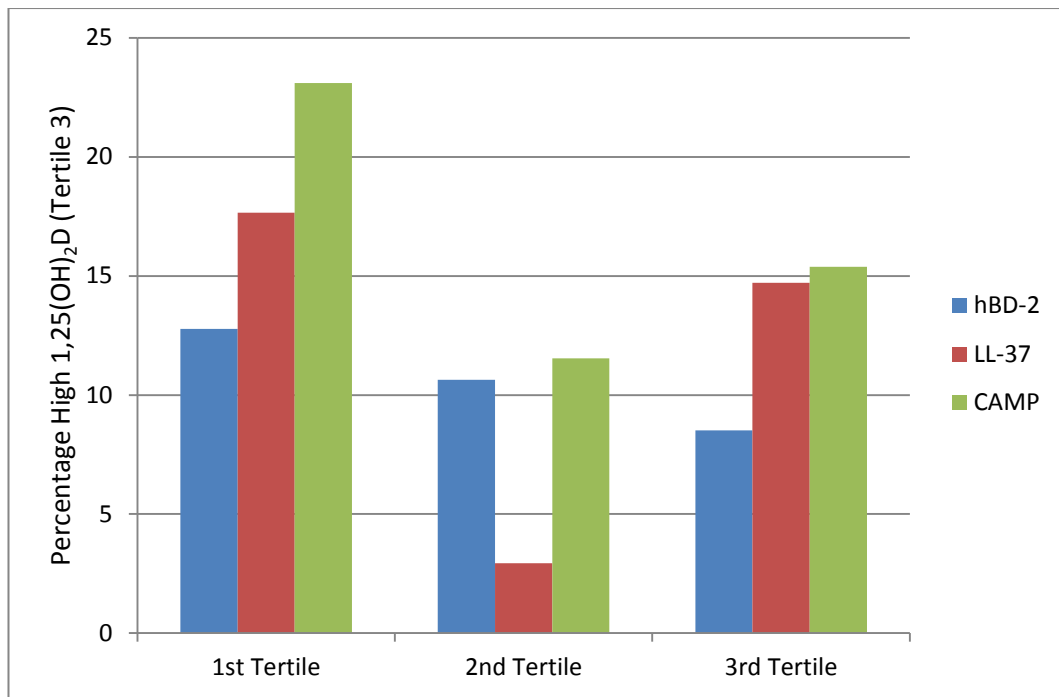
were then compared to the AMP tertile levels of LL-37, hBD-2 and CAMP (Figure 27, Figure 28, Figure 29).



**Figure 27 Relationship between percentage of low serum 1,25(OH)<sub>2</sub>D (Tertile 1) and the serum concentrations of hBD-2, LL-37 and CAMP**

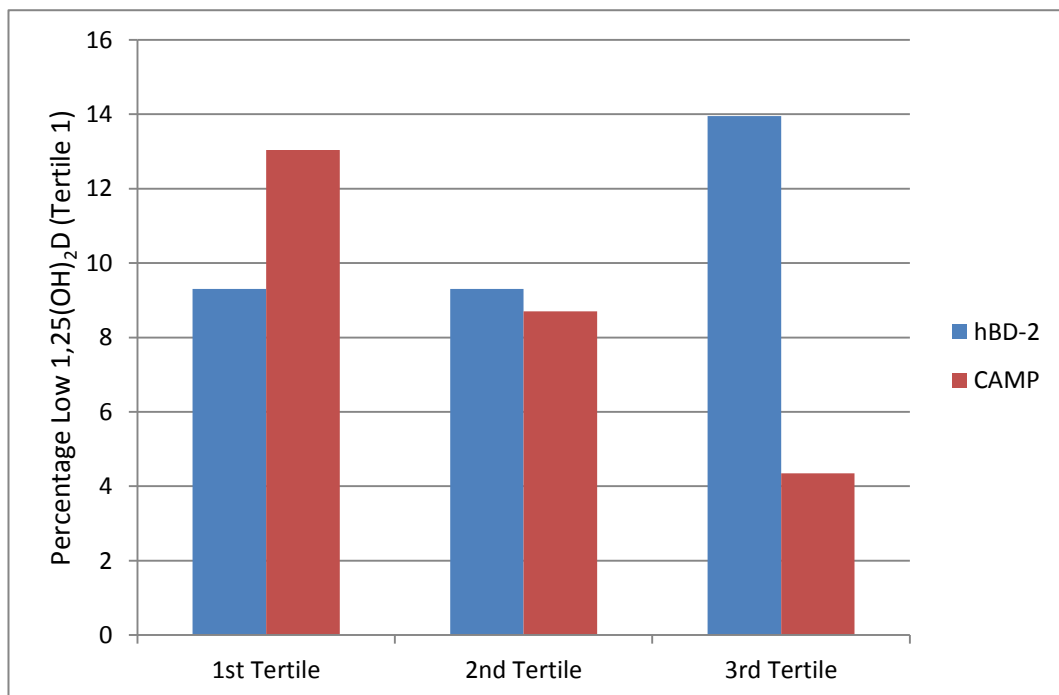


**Figure 28 Relationship between percentage of moderate serum 1,25(OH)<sub>2</sub>D (Tertile 2) and the serum concentrations of hBD-2, LL-37 and CAMP**

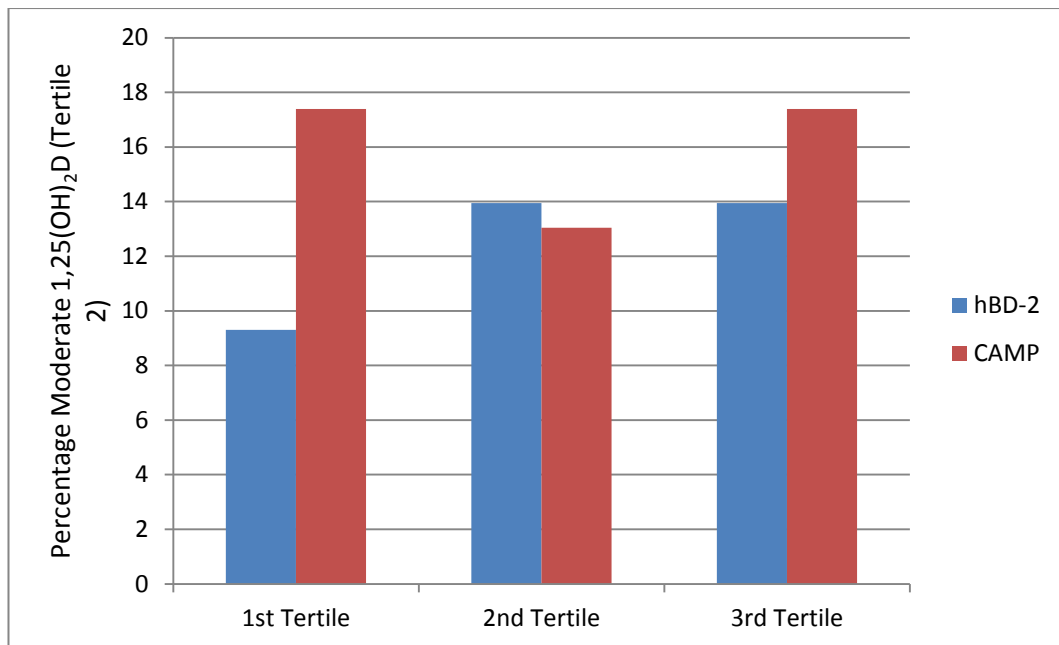


**Figure 29 Relationship between percentage of high serum 1,25(OH)<sub>2</sub>D (Tertile 3) and the serum concentrations of hBD-2, LL-37 and CAMP**

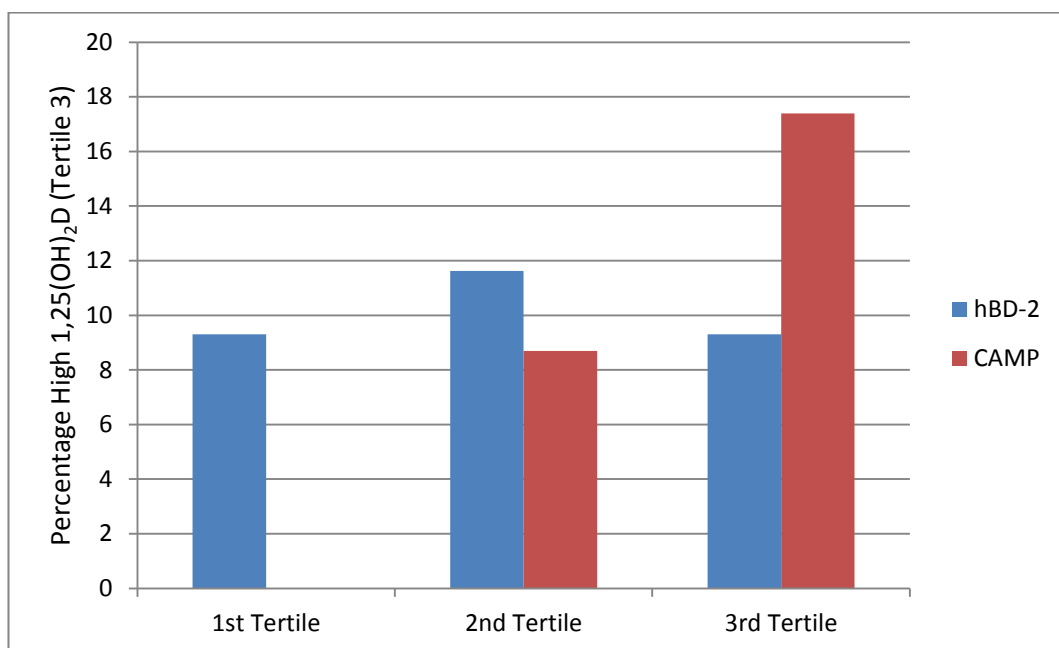
The tertile values of 1,25(OH)<sub>2</sub>D in sputum were then compared with the AMP sputum levels (Figure 30, Figure 31 & Figure 32). Only hBD-2 and CAMP has measured sputum levels available for analysis.



**Figure 30 Relationship between percentage of low sputum 1,25(OH)<sub>2</sub>D (Tertile 1) and the sputum concentrations of hBD-2 and CAMP**



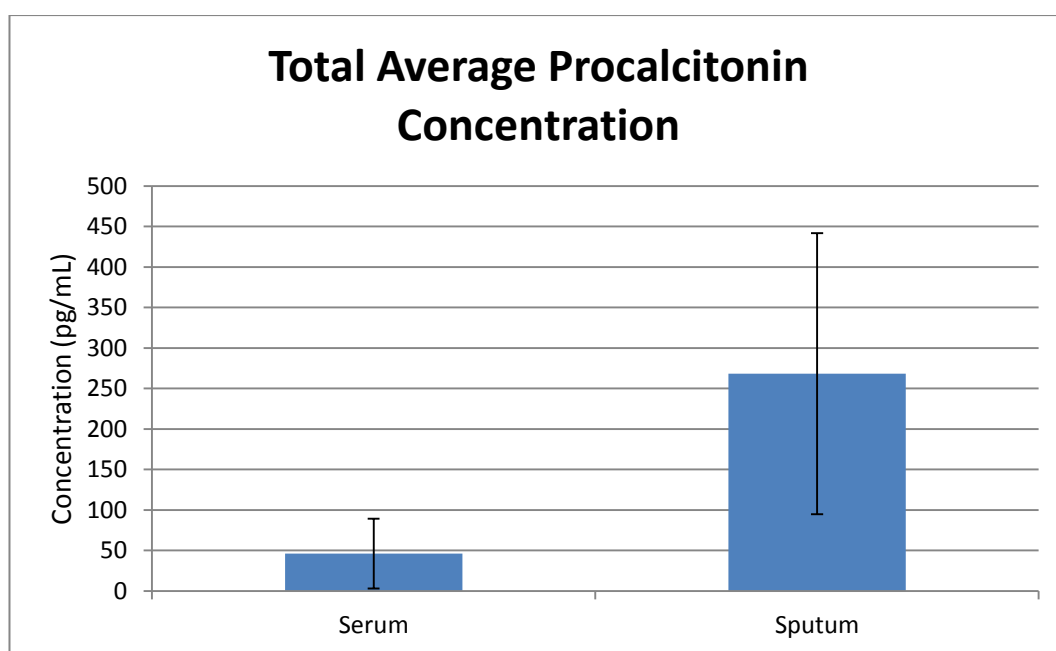
**Figure 31 Relationship between percentage of moderate sputum 1,25(OH)<sub>2</sub>D (Tertile 2) and the sputum concentrations of hBD-2 and CAMP**



**Figure 32 Relationship between percentage of high sputum 1,25(OH)<sub>2</sub>D (Tertile 3) and the sputum concentrations of hBD-2 and CAMP**

### 3.1.8 Procalcitonin Concentrations

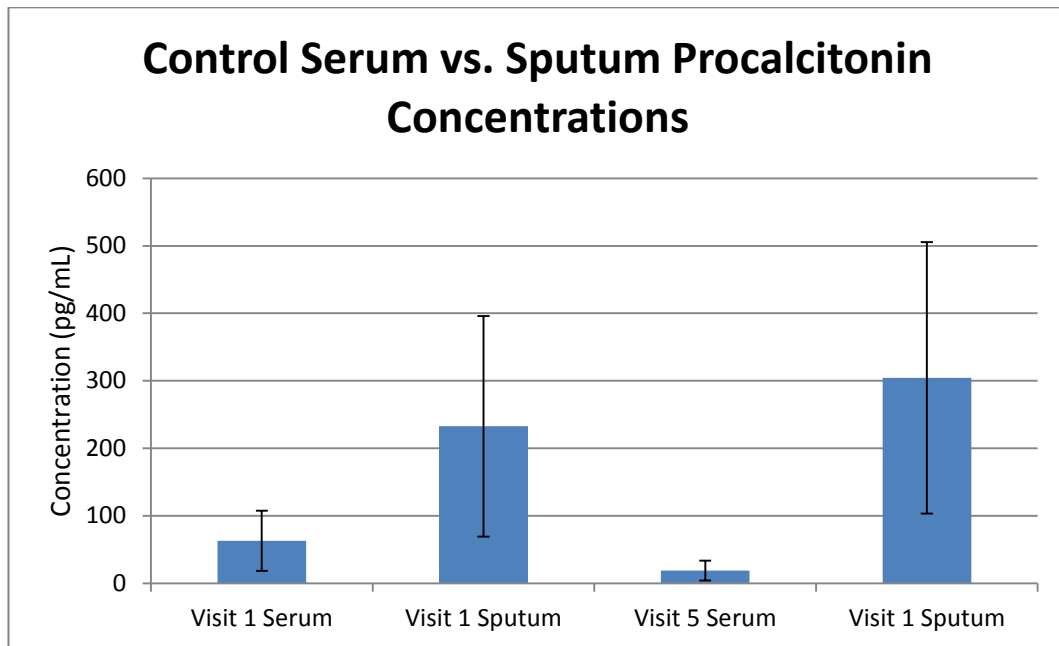
The total mean serum concentration was 62.88pg/mL (range=8.22-733.08pg/mL, n=41) and the median 28.06pg/mL (0.02806ug/L). The removal of one major outlier (visit 5 serum sample) changed the mean to 46.13pg/mL (range=8.22-179.79pg/mL, n=40). The one major outlier in this data was for a visit five serum sample, which had a recorded PCT level of 733.08pg/mL (0.73308μg/L). This level is over 0.5ug/L which is commonly used in studies to indicate the presence of a strong bacterial infection that should be administered antibiotics [148]. The total mean sputum PCT concentration was 268.17pg/mL (range=60.64-697.48pg/mL, n=49) and the median 211.39pg/mL (0.21139ug/L). A t-test reveals a high significance ( $p=0.000000007$ ) between serum PCT and sputum PCT as seen on Figure 33.



**Figure 33 Graph showing total average PCT concentration in serum\* and sputum of bronchiectasis patients (\*outlier omitted)**

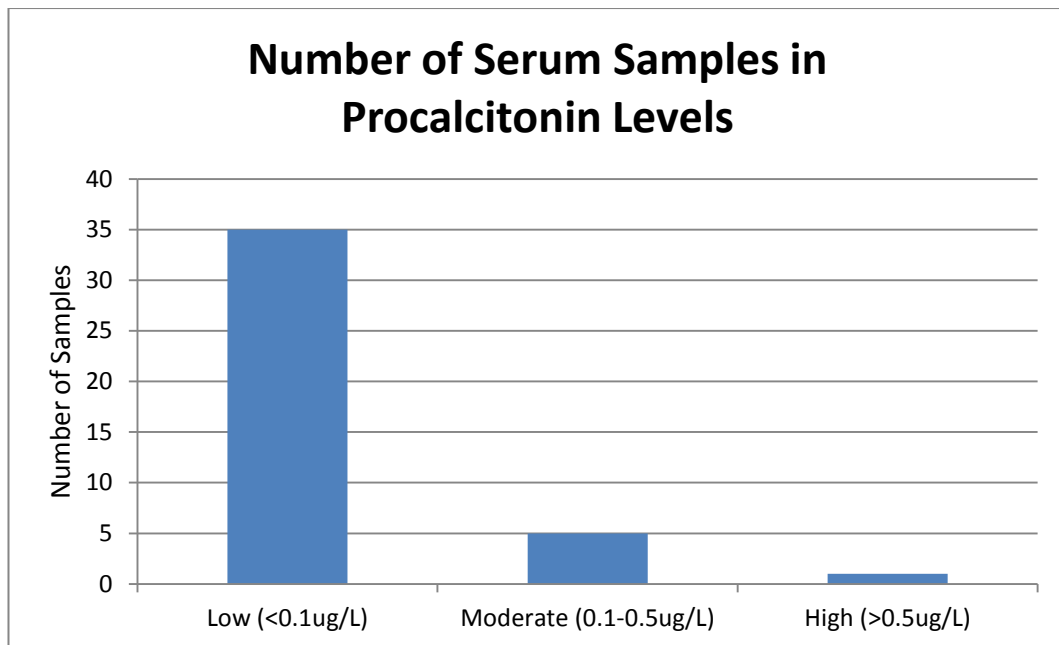
Only the baseline levels (Visit 1 & 6) were used for determining if there is any significant difference between the serum and sputum PCT concentrations. These two stages serve as the controls for their respective period. P-values were determined using a two-tailed Paired T-Test. There was a significant difference

found between serum and sputum levels for both baselines visit one ( $p=0.01$ ,  $n=11$ ) and visit six ( $p=0.04$ ,  $n=5$ ) further supporting the overall findings. The mean values for these control periods can be seen below on the graph. This graph further emphasises the difference between serum and sputum PCT concentrations (Figure 34).



**Figure 34 Graph of control (visits 1 and 6) serum vs sputum PCT concentrations**

Samples were broken down into predetermined cut-off levels of PCT. The lowest PCT level indicating no bacterial infection was 0.1ug/L (100pg/mL). A total of 35 out of 41 samples (85.37%) fell into this low PCT level. The next most prevalent group (12.20%) was moderate PCT levels (between 0.1-0.5ug/L or 100-500pg/mL) indicating a possible bacterial infection. Only one individual (2.44%) presented with a high PCT level above 0.5ug/L (500pg/mL) strongly indicating the presence of a bacterial infection.



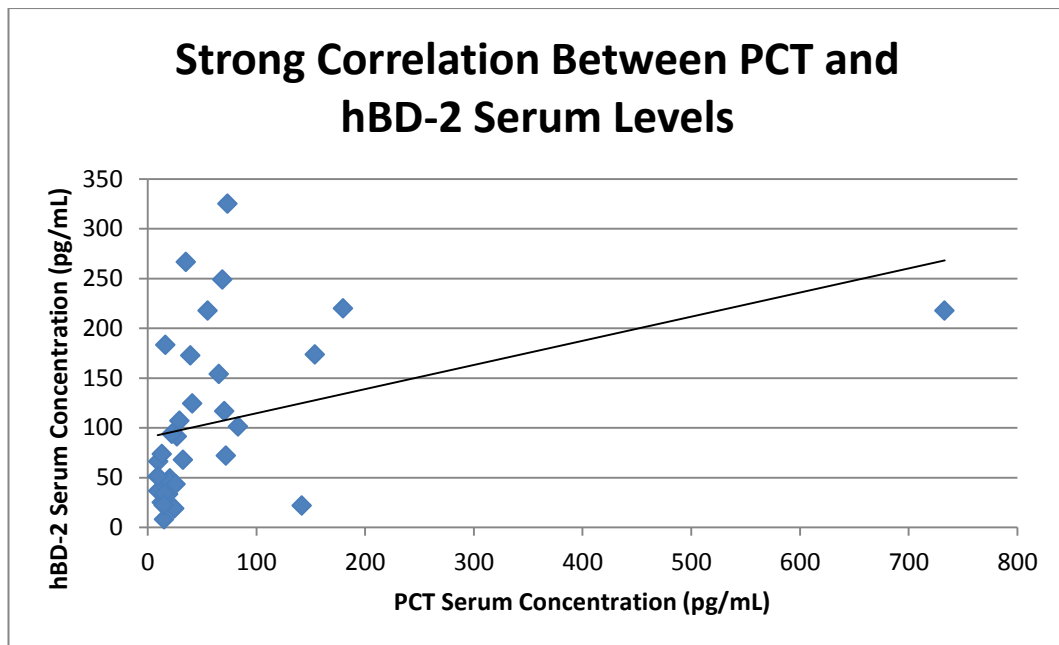
**Figure 35 Graph showing the breakdown of samples into predetermined PCT cut-off points**

### **3.1.8.1 Procalcitonin Correlation With Other Analytes**

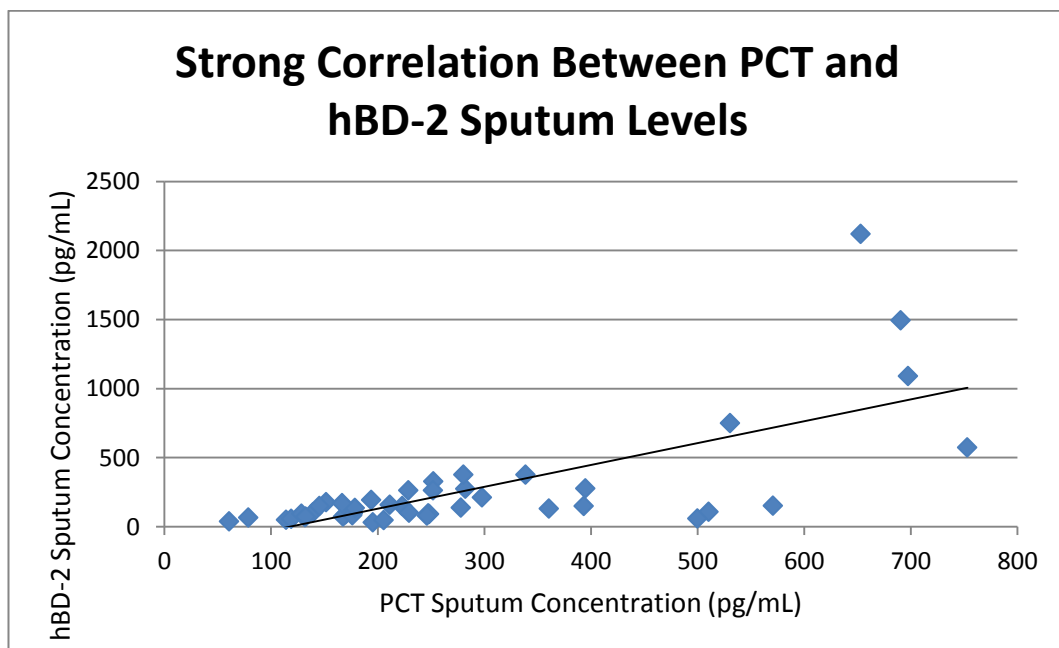
Spearman's non-parametric correlations were used to determine if there was any relationship between serum or sputum PCT and the other measured analytes.

Cathelicidin, 25(OH)D and 1,25(OH)<sub>2</sub>D only showed weak correlations with PCT serum or sputum levels. Strong positive correlations were found between PCT and hBD-2 in both serum ( $\rho=0.607$ ,  $n=33$ ) and sputum ( $\rho=0.671$ ,  $n=44$ )

bronchiectasis samples as can be seen observed in their respective scatterplots below (Figure 36 & Figure 37).



**Figure 36** A strong positive correlation exists between PCT and hBD-2 serum concentrations



**Figure 37** A strong positive correlation exists between PCT and hBD-2 sputum concentrations



### 3.1.9 Tiotropium Treatment Effect Analysis

#### 3.1.9.1 25-hydroxyvitamin D

No significant difference was found across the two crossover periods of the ROBUST trial. A Paired T-test showed no significance in serum concentrations between visits 1-5 ( $p=0.10$ ) or visit 6-9 ( $p=0.07$ ). The same for sputum concentrations, visit one to five ( $p=0.34$ ) and visit six to nine ( $p=0.52$ ).

#### 3.1.9.2 1,25-dihydroxyvitamin D

A significant difference was found across the both of the crossover periods of the ROBUST trial. A Paired T-Test has shown a significant difference in serum concentrations across both crossover periods, visit 1-5 ( $p=0.0028$ ) and visit 6-9 ( $p=0.000484$ ) of the clinical trial (Figure 38). No significance was identified across sputum concentrations. Only paired data was included in the T-Test, so some previously included samples excluded.

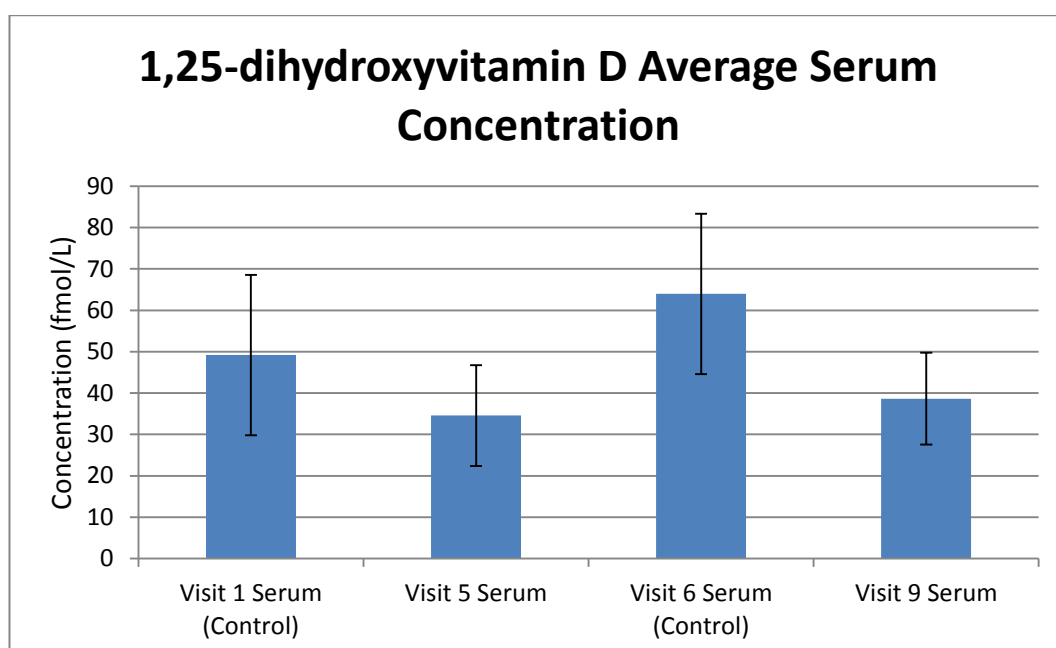


Figure 38 Graph of 1,25(OH)<sub>2</sub>D average serum concentration for whole clinical trial

### 3.1.9.3 Cathelicidin

For the Hycult LL-37 ELISA kit, a Paired T-Test showed no significance was across visits 1-5 serum ( $p=0.9500$ ) concentrations. A  $p$ -value of less than 0.05 was found across visit 1-9 ( $p=0.0041$ ) serum levels but the sample number was very limited ( $n=4$  pairs). Limited paired samples available to carry out Paired T-Test on the USCNK CAMP ELISA. No significant difference was found ( $p=0.125$ ) between CAMP serum levels across visits 1-5. Not enough sputum pairs available for sputum Paired T-test analysis.

### 3.1.9.4 $\beta$ -defensin 2

Using a Paired two-tailed T-Test (Table 7) there was no identified significant differences in hBD-2 concentrations across each half (Visit 1-5 and Visit 6-9) of the clinical trial in serum or sputum (Table 7). Only data that were paired were analysed, some previously used samples therefore were excluded. The  $p$ -values once this outlier was excluded from the Paired T-Test can be seen in brackets in Table 7, but still no significance. The mean values for these control periods can be seen on the graph.

**Table 7 Paired T-test results for hBD-2 concentrations**

	<b>Serum</b>		<b>Sputum (removed outlier)</b>	
	<b>Visit 1/5</b>	<b>Visit 6/9</b>	<b>Visit 1/5</b>	<b>Visit 6/9</b>
p-value	0.49	0.83	0.18 (0.18)	0.56 (0.12)
No. of Pairs	14	11	11 (10)	11 (10)

### 3.1.9.5 Procalcitonin

Using a paired two-tailed T-Test there was no identified significant differences found in PCT concentrations across each crossover half (visit 1-5 and visit 6-9) of the clinical trial, in serum or sputum. Only data that were paired were analysed, some previously used samples therefore were excluded. Statistical analysis of PCT samples was problematic due to difficulty with the kit in obtaining reliable

duplicates. No controls were provided with the ELISA kit. Therefore the number of paired samples for the Paired T-Tests is severely decimated.

**Table 8 Paired T-test results for PCT concentrations**

	<b>Serum</b>		<b>Sputum</b>	
	<b>Visit 1/5</b>	<b>Visit 6/9</b>	<b>Visit 1/5</b>	<b>Visit 6/9</b>
p-value	0.43	0.99	0.06	0.34
No. of Pairs	9	2	7	8

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# Chapter Four: Discussion

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## 4.1 Tiotropium Treatment Effect

The samples used were collected as part of a clinical trial investigating the usefulness of Tiotropium for the treatment of non-cystic fibrosis bronchiectasis. In this double-blind crossover study, the participants were receiving Tiotropium for six months and a placebo for the other six months. Samples were collected at the onset and completion of each crossover period.

Any significant differences found across this twelve month period in analyte concentration may indicate potential treatment effects. Paired T-tests were used to determine if any significant differences in analyte concentration occurred across each crossover period. No significant differences were detected for PCT, hBD-2 and 25(OH)D<sub>3</sub> serum or sputum concentrations across the twelve month trial. The use of a Paired T-test for cathelicidin was limited due to lack of paired samples. For the first crossover period, serum LL-37 and CAMP revealed no significant difference. A statistically weak difference ( $p=0.000484$ ) was found across visits six to nine for serum LL-37 but this was from a limited sample size ( $n=4$ ). A significant difference ( $p=0.0028$  and  $p=0.000484$ ) was found across both crossover periods for serum 1,25(OH)<sub>2</sub>D<sub>3</sub>. Seeing as this happened across both crossover periods it cannot be as a direct result of Tiotropium treatment. Potentially these observed differences are due to expected seasonal fluctuations in vitamin D concentrations. No differences were identified for 1,25(OH)<sub>2</sub>D in sputum.

Our findings suggest that during this twelve month period there were no substantial identified differences in the analytes. Therefore we can declare that treatment with Tiotropium does not appear to influence our results. Unfortunately due to the ROBUST clinical trial still being in progress, the randomisation codes still remain blind.

## 4.2 Vitamin D

Serum 25(OH)D concentration is the normal clinical measure of vitamin D status. Our research showed that sub-optimal vitamin D levels ( $<75\text{nmol/L}$ ) are commonplace in bronchiectasis patient serum samples (52.9%). The median 25(OH)D<sub>3</sub> serum level of  $71.31\text{nmol/L}$  for this stable non-cystic fibrosis bronchiectasis patient cohort, is not consistent with the only other study of VDD in bronchiectasis. During the course of this research, a paper was published by Chalmers *et al.*, that reported a median 25(OH)D level of  $24.7\text{nmol/L}$  with a total of 50% of their cohort being classed as vitamin D deficient ( $<25\text{nmol/L}$ ), compared to 12% of their control group [43]. Our research only indicated a VDD rate of 27.45% based on a different cut-off for VDD ( $<50\text{nmol/L}$ ). Our findings reject our hypothesis of VDD being prevalent in bronchiectasis patients.

The potential reasons for this inconsistency could be the use of vitamin D supplementation. In the Chalmers *et al.* study a total of 16.9% of patients were excluded due to vitamin D supplementation use [43]. This was not assessed in our patient cohort. The timing of the sample collection in this clinical trial meant that there was an underrepresentation of samples collected during the winter months. The majority of samples were taken in spring and early autumn. Potentially the data therefore is not providing an accurate annual representation of vitamin D levels.

Our 25(OH)D<sub>3</sub> serum levels ( $71.31\text{nmol/L}$ ) are higher than those reported for other respiratory diseases. In community acquired pneumonia the median 25(OH)D serum level was  $54\text{nmol/L}$  [107]. But interestingly in patients identified as receiving vitamin D supplements, the median increased to  $85\text{nmol/L}$  which is more consistent with our findings. Their study used the same cut-off points for VDD as our study ( $<50\text{nmol/L}$ ), with severe VDD defined as  $<30\text{nmol/L}$ . Their total VDD rate was 44% compared to our 27.45%, and 15% compared to our 7% for severe VDD. Our data was also higher than the reported 25(OH)D serum levels in COPD  $19.9\text{ng/mL}$  ( $49.67\text{nmol/L}$ ); VDD was associated with the severity of disease in this instance [133].

The median 25(OH)D<sub>3</sub> sputum level was 14.40nmol/L, which was significantly lower ( $p=0.0000$ ) than the serum 25(OH)D level. The same is true for 1,25(OH)<sub>2</sub>D serum and sputum concentrations ( $p=0.0000$ ). These lower vitamin D metabolite sputum levels may be a result of transport limitations [117]. It is thought that because vitamin D metabolites have a high affinity for their carrier proteins (eg. DBP, albumin), with over 99% of vitamin D metabolites being bound within the blood [116], this may ultimately affect their transportation into airways [117]. Potentially concentrations are also limited by receptor binding affinity or the active transport of these metabolites into the epithelial cells [155]. No data is currently available on the presence of vitamin D metabolites in sputum for comparison. But lower concentrations of 25(OH)D and 1,25(OH)<sub>2</sub>D have been reported in BAL fluid (localised) compared to their respective serum counterparts [117].

The vitamin D metabolite, 1,25(OH)<sub>2</sub>D<sub>3</sub>, has been included in this study as previous studies focused on 25(OH)D have failed to show an association with AMP concentrations [43, 107]. Typically 1,25(OH)<sub>2</sub>D is not used to assess vitamin D status, as it has a very short half-life (approximately 4 hours) and its renal production is tightly regulated for bone health. 1,25(OH)<sub>2</sub>D is the active form and is the vitamin D metabolite that has been shown *in vitro* to directly upregulate various target genes, including AMP's. Its non-renal synthesis in bronchial epithelial cells and immune cells is possible due to the required enzymatic machinery being present. It is therefore hypothesized that an *in vivo* association between 1,25(OH)<sub>2</sub>D and AMP's concentrations may exist.

The median 1,25(OH)<sub>2</sub>D<sub>3</sub> serum concentration was 48.08pmol/L. But no standardised levels of 1,25(OH)<sub>2</sub>D have been determined. Two studies have reported slightly higher 1,25(OH)<sub>2</sub>D levels in serum compared to our study. In a healthy Danish population a reported level of 29.0pg/mL (69.6pmol/L) [153] and the other study a level of 23.6pg/mL (56.64pmol/L) in their control group [154]. This would indicate that our reported 1,25(OH)<sub>2</sub>D serum concentrations are lower than those reported in healthy cohorts.

Our study revealed a moderately strong positive correlation using Spearman's non-parametric correlation, between serum 25(OH)D<sub>3</sub> levels and serum 1,25(OH)<sub>2</sub>D<sub>3</sub> levels (rho=0.6910). This indicates that the level of serum 1,25(OH)<sub>2</sub>D is most likely dependent on its substrate 25(OH)D concentration. But remarkably no correlation was found between 1,25(OH)<sub>2</sub>D<sub>3</sub> sputum levels and serum 25(OH)D<sub>3</sub> levels (rho=-0.1560). This may potentially indicate that these localized 1,25(OH)<sub>2</sub>D levels are under the control of some unknown local regulation, for example, growth factors or cytokines. Non-renal 1,25(OH)<sub>2</sub>D is not under the regulation of PTH like the renal form. But the active local concentrations are not fully dependent on their substrate concentration as expected.

Evaluation of 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations has revealed strong localised effects. The local concentrations of these metabolites are independent from the circulating serum vitamin D metabolites concentrations. Sub-optimal or insufficient vitamin D levels are prevalent in non-cystic fibrosis bronchiectasis. This may indicate causality, but more likely due to the fact that respiratory conditions lead to a reduced outdoor activity and therefore reduced sun exposure. Hypovitaminosis D is a consequence of living with a respiratory condition and not a causative factor [49, 108, 133, 156].

### 4.3 Vitamin D and Antimicrobial Peptides

VDRE's have been discovered in the promoter region for both of the genes for cathelicidin and hBD-2 [88]. It is known that the VDR-1,25(OH)<sub>2</sub>D-RXR complex is capable of regulating the expression of these genes via these VDRE's, as shown in multiple *in vitro* studies [87, 88]. The hypothesis thereby exists that individuals with lower vitamin D levels would correspondingly have decreased AMP levels. This effect though has yet to be proven *in vivo*. The best way to detect any relationships between the vitamin D metabolites and AMP's is using Spearman's non-parametric correlations.

All comparisons between LL-37, CAMP and hBD-2 concentrations with 25(OH)D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub> serum and sputum concentrations, have revealed very weak correlations. Except for the moderate positive correlation found between

CAMP sputum levels and 1,25(OH)<sub>2</sub>D sputum levels ( $\rho=0.497$ ). One study has reported similar findings with a moderate association being found between hCAP18 and 1,25(OH)<sub>2</sub>D plasma levels but again a weak correlation between hCAP18 and 25(OH)D [106].

hBD-2 and LL-37 failed to support the hypothesis of lower vitamin D levels being associated with correspondingly low AMP levels. This is evident when data was log-transformed and broken down into tertiles (Section 0). LL-37 and hBD-2 showed no trends with 25(OH)D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub> cut-off points or tertiles. Only CAMP sputum showed this trend with 1,25(OH)<sub>2</sub>D sputum, with only the two higher CAMP tertiles (2 and 3) being found in the highest 1,25(OH)<sub>2</sub>D tertile (tertile 3) (Figure 32). This further emphasises the above finding of a moderate positive association ( $\rho=0.497$ ) between CAMP sputum and 1,25(OH)<sub>2</sub>D<sub>3</sub> sputum.

But other previous studies have also failed to find any association either LL-37 or hBD-2 with serum 25(OH)D. The suggestion was made that LL-37, the active peptide fragment, may not be the most suitable measure. The prepropeptide, which is normally cleaved by enzymes to release active LL-37, may provide more insight [107]. Our data supported this as no correlations were identified between LL-37 and vitamin D metabolites. These results indicate that focusing on the active form 1,25(OH)<sub>2</sub>D may be more valuable in identifying these associations with AMP's *in vivo*.

The recent Chalmers *et al.* paper also failed to find an association between serum 25(OH)D and LL-37 sputum concentrations in bronchiectasis patients. In fact this paper actually identified higher LL-37 sputum levels in VDD individuals [43]. Interestingly, our data revealed a trend between the highest serum LL-37 tertile (Tertile 3) and VDD. LL-37 serum (Tertile 3) was the largest group in VDD (18.18%) and the largest represented grouping overall (Figure 24). Unfortunately that paper failed to report their actual mean or median sputum LL-37 values so unable to compare these findings [43]. This suggests that vitamin D effects *in vivo* have little effect on LL-37 level. The relationship between DBP in the airway and neutrophil mediated inflammation may be a more important mechanism in the



link between vitamin D and disease severity. High levels of DBP in sputum are associated with airway inflammation and disease severity in bronchiectasis [43].

The *in vitro* studies of 1,25(OH)<sub>2</sub>D treatment on various cell-lines showed strong upregulation of CAMP expression but only a modest effect on hBD-2 levels [88]. Consequently it is not surprising that hBD-2 failed to show any association with the vitamin D metabolites *in vivo*. The finding of a positive correlation between CAMP sputum levels and 1,25(OH)<sub>2</sub>D sputum levels *in vivo* supports the findings of the *in vitro* experiments.

## 4.4 Cathelicidin

Two separate ELISA kits were used to analyse cathelicidin levels. The median serum LL-37 level was 50.44ng/mL. This is nearly identical to a reported LL-37 median serum level in tuberculosis patients of 49.5ng/mL [157]. In a similar study, that was using the same ELISA kit, they reported a median level of 69ng/mL in community-acquired pneumonia [107]. Our reported serum LL-37 value is slightly lower but still fairly consistent. The only valid control LL-37 serum concentration available was a prior study to the above tuberculosis study. The study reported a mean serum 27.21ng/mL in a healthy outpatient cohort [105]. Therefore it appears that serum LL-37 is elevated in a number of these chronic respiratory conditions, including in our bronchiectasis cohort. Potentially LL-37 could act as an indicator of an amplified immune activation involved in the chronic infection progression of these conditions [157].

The Leow *et al.*, 2001 study identified that cathelicidin concentrations did not tend to drop after an acute admission. Follow-up levels remained similar to admission levels [107]. Our study found no statistical robust difference ( $p=0.950002$ ) between LL-37 between visit one and five samples. Patient samples tended to remain consistent over the course of the clinical trial. This indicates that cathelicidin levels may be influenced by more than an acute exacerbation; other factors may be involved. Again no standardised normal serum level of LL-37 has been established, so without a control population it is difficult to detect if directly compare if levels are elevated or not.

Interestingly, no correlation was found between the LL-37 levels and the CAMP levels ( $\rho=-0.1413$ ). This may be because the two kits are measuring different cathelicidin components. Unfortunately the CAMP ELISA kit failed to state exactly what it was measuring. It is unknown if the USCNK assay was measuring the precursor of cathelicidin (hCAP18) or the active peptide component LL-37, or both. The median CAMP serum level was 412.67ng/mL and the median CAMP sputum level was 55.73 $\mu$ g/mL, showing a 106-fold increase. The CAMP sputum level was statistically higher ( $p=0.00000001$ ) than the CAMP serum level. Two sputum samples were run on the USCNK LL-37 kit, these samples showed a 39-fold and 72-fold increase over their respective serum equivalent. This data indicates that serum cathelicidin levels are not representative of what is happening at localised sites in bronchiectasis.

Most often hCAP18 or LL-37 levels are measured in plasma, which is not directly comparable with serum levels. There is a scarcity of valid comparable data for our study. Reported mean plasma hCAP18 levels in control groups are 1180 $\pm$ 200ng/mL in a Denmark volunteers [98] and 650 $\pm$ 343ng/mL in control patients of a haemodialysis study [106]. These values appear to fit better with our CAMP serum mean of 569.12ng/mL, suggesting that this ELISA kit may in fact be measuring cathelicidin precursor levels. Our mean is lower than both of these control groups.

There is a lack of data investigating localised cathelicidin levels compared to circulating levels. In BAL fluid, the measured LL-37 levels are lower than the serum LL-37 concentrations [117]. This is opposite to the high localised levels found in sputum of our bronchiectasis cohort. Higher localised cathelicidin levels are expected due to their natural role in host defense and consistent with bronchiectasis being a localised condition. One study has investigated sputum LL-37 levels in bronchiectasis but unfortunately this paper failed to report their mean or median concentration [43]. In COPD, LL-37 in induced sputum has been shown to be elevated compared to two control groups (healthy smokers and healthy non-smokers) [158]. hCAP18 has been shown in both CF and COPD to be elevated in induced sputum when compared to serum counterparts and control groups [159, 160]. Our median CAMP sputum level was 55,728.44ng/mL

(55.72ug/mL); still higher than their reported level of 79,623pg/mL in CF and 75,262pg/mL in COPD [159].

Investigations have indicated a possible synergistic relationship between LL-37 and hBD-2 [94, 95]. This concept has been supported by animal sepsis models in which hCAP18 have been capable of initiating the release of inducible defensins [161, 162]. The use of Spearman's non-parametric correlations revealed potential relationships between cathelicidin and hBD-2. A strong positive correlation ( $\rho=0.633083$ ) was found between hBD-2 serum and CAMP sputum levels. This indicates that as the levels of CAMP increase in the sputum, so do the hBD-2 levels in circulation. A moderate negative correlation ( $\rho=-0.44273$ ) was found between serum LL-37 and serum hBD-2, therefore as the level of one increases the level of the other decreases. All the other correlations between cathelicidin and hBD-2 serum or sputum revealed weak correlations. These two potential relationships reveal a possible interaction between these two AMPs against infection. More research is needed into further refining these relationships.

Additionally, other elements may be contributing to the regulation of cathelicidin levels. Cathelicidin, like other AMPs, have numerous functions in addition to its antimicrobial properties. Cathelicidin is known to recruit important immune cells, neutrophils, monocytes, leucocytes and T cells, to sites of inflammation [117]. High concentrations of sputum LL-37 has been correlated with *Pseudomonas aeruginosa* colonization [117]. In future studies it may be worthwhile to culture sputum and look at the pathogens isolated.

The overall cathelicidin findings indicate elevation of serum LL-37 levels in bronchiectasis when compared to control cohorts. But the CAMP serum levels appear to be decreased in bronchiectasis, but there are only plasma hCAP18 control levels available for comparison. In general, cathelicidin levels remain steady in these stable bronchiectasis patients. Higher sputum levels have supported the notion of elevated localised cathelicidin levels in bronchiectasis. This finding is consistent with bronchiectasis being a localised disease that is characterised by frequent acute pulmonary exacerbations.

## 4.5 $\beta$ -defensin 2

The finding of a significant difference ( $p=0.0044$ ) between serum and sputum levels of hBD-2 is not surprising. The median serum level was 72.88pg/mL compared to the median sputum level of 136.84pg/mL, showing that hBD-2 levels are elevated in sputum of bronchiectasis patients. The AMP hBD-2 is produced chiefly by epithelial cells and is known to be secreted by the bronchial epithelial cells. Therefore, one would expect that the serum concentration would not accurately represent local hBD-2 levels in bronchiectasis. But contrastingly other studies have found a good correlation between both plasma (systemic) and BAL fluid (localised) defensin levels in pneumonia [163, 164]. Unfortunately, there are currently no studies on hBD-2 levels in bronchiectasis.

The existing studies on hBD-2 levels in various respiratory conditions provide conflicting results. Again there is no consensus on normal healthy hBD-2 levels. In community-acquired pneumonia the median serum hBD-2 level was 262pg/mL [107] which is considerably higher than our reported value (72.88pg/mL) for bronchiectasis. In COPD, the level of hBD-2 in the sputum supernatant fell below the lowest limit of detection ( $<8$ pg/mL) for the ELISA. The two control groups for this study reported mean levels of 98pg/mL in healthy smokers and 74pg/mL in never-smokers [165]. Our mean sputum (246.71pg/mL) finding is substantially higher than these two control groups.

Panbronchiolitis (DPB) is a respiratory disease very similar to bronchiectasis, and is in fact one of its identified aetiologies. DPB is similar to bronchiectasis in a number of ways including the frequent periods of infection, chronic inflammation and colonization by *Pseudomonas aeruginosa* and *Haemophilus influenzae* in the sputum. Even with these similarities their findings show again the opposite to our study, with higher systemic hBD-2 level (plasma-167.6pg/mL) than in BAL fluid (71.5pg/mL) in DPB patients; both significantly higher than their control groups [166].

All these inconsistencies indicate that other factors must be influencing hBD-2 levels. Stronger stimulation of hBD-2 expression is reported when 1,25(OH)<sub>2</sub>D is

in the presence of IL-1 $\beta$  [167]. This signalling pathway works via TLR1/2 (PRP) to induce expression of IL-1 $\beta$  in monocytes. The signalling by IL-1 $\beta$  to enhance 1,25(OH) $_2$ D's effect most likely due to binding by the transcription factor NF- $\kappa$ B to specific binding site in hBD-2 promoter [168]. Therefore analysing IL-1 $\beta$  concentrations might be beneficial.

One study on sputum hBD-2 levels in acute pneumonia revealed suppressed levels of hBD-2 in both sputum and pharyngeal washes in individuals who smoke or are ex-smokers [169]. Smoking therefore is linked to an increased susceptibility of an individual to infection. Knowing our patient cohorts smoking status may have enable further investigation into this association. The expression of hBD-2 has been also shown to be inducible during infections or inflammation. Even though hBD-2 is the most highly expressed defensin in the lung, there are multiple other human defensins that also may overlap in the same tissues. Possibly some defensins may be capable of substituting for hBD-2.

No conclusion can be made on the role of hBD-2 in the pathology of chronic respiratory tract infections. Our novel finding of higher localised levels of hBD-2 seems to be inconsistent with reports for other respiratory conditions. Being the only study so far investigating hBD-2 concentrations in bronchiectasis, further analysis is needed for confirmation. The possibility exists that sputum samples are potentially contaminated by saliva during sputum collection, in which high defensin levels are present.

Both of the AMP's, cathelicidin and hBD-2, showed statistically elevated concentrations in the sputum compared to serum. AMP's normal role is to function in the innate immune system to provide host defence. Bronchiectasis is known to be a localised rather than systemic condition; therefore one would not expect a flowover effect to be seen in the serum.

## **4.6 Procalcitonin**

Bronchiectasis patients frequently experience reoccurring LTRI's [3]. Preventing these exacerbations is vital to increasing the patient's management of the

condition, quality of life and decreasing the disease progression. Current treatments are based on extrapolations for the treatment of other respiratory conditions. Finding a suitable inflammatory marker for bronchiectasis may aid in guiding antibiotic treatment. Currently no suitable systemic inflammatory marker exists for these exacerbations in bronchiectasis.

Studies suggest that stable patients with non-cystic fibrosis bronchiectasis have consistently elevated systemic markers of inflammation. This has been reported with two systemic inflammatory markers, CRP and ESR [139]. PCT is a new inflammatory marker that has proven useful in the detection of bacterial infections [141]. Due to these frequent bacterial exacerbations in bronchiectasis, one would expect both systemic and local inflammatory markers to be elevated, even during a stable state [137, 140]. Therefore it was expected that both PCT serum and sputum levels would be elevated in this patient cohort.

Results show that serum PCT levels appear to be very low and therefore not consistently elevated in stable bronchiectasis patients. The median PCT level reported in COPD was 0.096ug/L [142] and 0.57ug/L in pneumonia patients [149]. The median PCT serum value in this study was only 0.028ug/L for stable bronchiectasis patients. This value aligns bronchiectasis more closely to COPD than pneumonia. Both COPD and bronchiectasis are more localized diseases, with infection and inflammation generally being limited to the mucus linings of the respiratory tract.

The only other study that has reported PCT serum levels in bronchiectasis revealed a median of 0.055ug/L [138]. Our reported median serum PCT level is slightly lower than this. Both these values are under 0.1ug/L, which is generally used to indicate no bacterial infection (clinical sepsis) and no requirement for antibiotics. A total of 14.63% of our samples recorded PCT levels above this 0.1ug/L threshold; compared to the only other study which had 21.1% above [138]. But potentially these results are influenced by a high proportion of patients in that study receiving prophylactic antibiotics.

Without comparing to actual acute exacerbations a conclusion cannot be made on the suitability of PCT in managing bronchiectasis exacerbations. Some patients did show higher PCT levels at specific visits but not consistently higher over the 54 weeks. It would have been advantageous to match these higher PCT levels with actual acute exacerbations or sputum bacteriology. But it is clear that PCT is not constitutively elevated in the serum of stable bronchiectasis patients. Potentially the reason that serum PCT is not consistently elevated is that all exacerbations may not be the result of bacterial infections, or these infections may be localized within the bronchial tissue.

PCT sputum levels are significantly higher ( $p=0.000000007$ ) than in serum which is consistent with bronchiectasis being a very localized rather than systemic condition. The median sputum PCT level is 0.21139ug/L (0.21139ng/mL) compared to the median PCT serum level of 0.02806ug/L (0.02806ng/mL). This significance was detected over both control periods (V1,  $p=0.01$  and V6,  $p=0.04$ ) as well as over the whole 54 weeks. Unpublished data supports these elevated PCT levels being present in sputum (2ng/mL) against serum ( $<0.5$ ng/mL) in bronchiectasis patients with acute exacerbations (Wong *et al.*, 2011). Unfortunately no published research is available on the function and concentration of normal PCT in sputum. Elevated PCT sputum levels are expected though, as sputum contains a higher density of both bacteria and inflammatory cells.

There was a major issue with the reproducibility of the PCT ELISA kit. Duplicates were difficult to obtain even though no dilutions were needed. Therefore the %CV required was increased to 40% to enable enough data to be used for statistical analysis. Even then a large number of samples could not be included in the results. These ELISA kits are generally set up to detect any abnormally high PCT levels. Many of the samples had very low OD values ( $>0.2$ ) which unfortunately places them at the most inaccurate point of the standard curve. The use of triplicates may have aided in obtaining more consistent results.

An interesting comparison would be between PCT and CRP levels in bronchiectasis during and after acute exacerbations. CRP is typically associated with systemic inflammation and PCT with bacterial infections. PCT has many

properties that make it more desirable, peaking at just over 8 hours from initial infection. PCT has been shown to have better specificity and sensitivity than CRP during an episode of bacterial infection [147]. An interesting strong positive correlation found was between both PCT and hBD-2 serum ( $\rho=0.607$ ) and sputum levels ( $\rho=0.671$ ). The potential mechanism behind this relationship is unknown and may warrant further investigation. The overall PCT findings show that serum PCT is not elevated in stable bronchiectasis patients, however is significantly elevated in localised respiratory levels.

## 4.7 Factors Influencing Vitamin D Levels

There are several complexities that make the direct analysis of vitamin D status challenging. Firstly is the difficulty in determining 25(OH)D cut-off points for deficiency. Most currently accepted vitamin D cut-offs are based on those required for bone health with no regard for vitamin D's pleiotropic actions. Another complicating issue when studying vitamin D is the numerous vitamin D metabolites that exist [60]. The commercial ELISA kits used in this research were based on the predominant vitamin D<sub>3</sub> metabolites. The use of ELISA kits capable of measuring both D<sub>2</sub> and D<sub>3</sub> together will improve the accuracy of results. Finally, there are many genes involved in the transport and metabolism of vitamin D. The DBP and VDR genes have been well researched and genetic variations have been linked to vitamin D bioavailability. These polymorphisms may ultimately effect 1,25(OH)<sub>2</sub>D's action on target genes.

Vitamin D is a lipophilic molecule and therefore relies on plasma proteins for transportation to target cells. At any one time up to 99% of vitamin D metabolites are bound [116]. The primary carrier is DBP, with smaller amounts being carried by albumin. Being bound to DBP greatly extends the circulating half-life of 25(OH)D [61, 116]. Studies have indicated that polymorphisms in the DBP gene (*GC*) may have an influence on the binding of vitamin D metabolites. Three common variants are *GC1F*, *GC1S* and *GC2*. The most well-known non-synonymous isoforms are the SNP's rs4588 and rs7041 [118]. The rs7041 (*TT* genotype) has been investigated in COPD and has been linked to lower 25(OH)D concentrations [133]. Variants in the *GC* gene (rs4588-AA allele) have also been



associated with an increased individual's response to vitamin D supplementation [170].

SNP's of the *GC* gene have not only been linked to lower levels of 25(OH)D but also to higher levels of DBP [118, 171]. DBP has many other functions including actin binding, macrophage activation, and neutrophil chemotaxis [118, 133]. These factors are important as bronchiectasis is regarded as a neutrophil-driven disorder [25]. Neutrophils are the predominant cell type isolated from the airway fluid of bronchiectasis patients [172]. Elevated numbers of neutrophils exist in stable bronchiectasis patients and increase further with an exacerbation [173].

The VDR is responsible for binding the ligand 1,25(OH)<sub>2</sub>D and forming a heterodimer with the RXR. This enables the binding to VDRE's on target genes. Polymorphisms within the VDR gene will influence association with target genes (eg. CAMP and hBD-2) rather than circulating 25(OH)D levels. There are five common polymorphisms *Cdx2*, *FokI*, *ApaI*, *TaqI* and *BsmI* [174]. The VDR polymorphisms *FokI* (*ff* genotype) and *TaqI* (*Tt* genotype) is associated with the risk lower respiratory tract infections in young Canadian children [175]. The *FokI* *f* allele results in a VDR protein that is longer and functionally results in a lower rate of VDR transcription [176]. This *f* allele has also been associated with risk of multi-drug resistant tuberculosis [177].

Another potential influential factor could be the active transport of 25(OH)D into the target cells. There are many other elements involved in the binding of 1,25(OH)<sub>2</sub>D including protein kinase A (upregulates) or protein kinase C (downregulates). These can therefore have an impact on target gene expression. Transcription factors are also required to help bind the VDR-1,25(OH)<sub>2</sub>D-RXR complex to VDRE's, for example, NF-κB [178]. Numerous additional factors need to be considered in the assessment of vitamin D concentrations.

## 4.8 Study Limitations

Unfortunately sample number had to be restricted due to the cost and availability of commercially available ELISA kits. The majority of samples obtained from Auckland Hospital contained insufficient sputum volume to carry out all ELISA tests. The use of these samples therefore did not allow a complete data set to be obtained for these patients. The small sample size limits complex statistical analysis of results. Results could have been strengthened by the use of a healthy control population for direct comparison. Currently little data is available regarding normal serum or sputum levels of cathelicidin, hBD-2, PCT and 1,25(OH)<sub>2</sub>D.

A lack of provided medical history makes the analysis of results challenging. Bronchiectasis patients often experience frequent reoccurring exacerbations, and it was unknown whether any individuals experienced acute exacerbations during the clinical trial. For example, one individual showed a high PCT level (733.08pg/mL), this is over the suggested level of 0.5ug/L used to indicate a severe bacterial infection. Pulmonary function (eg. %FEV<sub>1</sub>) gives an insight into severity of bronchiectasis; VDD and AMP levels have been correlated with disease severity previously. Studies have also shown associations between mortality and VDD [107] as well as hCAP18 levels and mortality [106]. Nutritional status is also a big contributor for vitamin D concentration.

In addition to these, smoking status may have provided some insight. Previously smoking has been associated with lower levels of hBD-2 in sputum [169]. Age and ethnicity are also known to be associated with VDD, both of which decrease the synthesis of the precursor (7-dehydrocholesterol) to vitamin D<sub>3</sub>. It is also unknown whether any individuals could be possibly taking vitamin D or calcium supplementation. The use of vitamin D supplementation could artificially be skewing the data. Both of these supplements are readily available without a prescription.

Some of the ELISA kits proved to be poor quality, especially the PCT and CAMP kits. This resulted in the %CV needing to be increased to allow enough data for

statistical analysis. Sputum proved rather difficult to work with in ELISA tests. Accurate duplicates were often difficult to obtain even though no sputum dilutions were being performed. Inter-assay variability is a major issue with commercial ELISA kits. Kits produced in different lots often produce dissimilar standard curves. Producing an average standard curve for each analyte may have been beneficial when inter-assay variability is detected. The vitamin D controls concentration was not provided; therefore they were only useful for assessing that there were no major errors with how the kit was run. Major issues with inter variability between laboratories has been frequently reported with vitamin D assays.

## **4.9 Conclusions and Future Research**

This study provides evidence for the uneven distribution of AMP's, vitamin D metabolites and procalcitonin, between systemic and localised respiratory levels in non-cystic fibrosis bronchiectasis. Both of the antimicrobial peptides, cathelicidin and  $\beta$ -defensin 2, showed elevated sputum levels compared to serum levels. This places the antimicrobial peptides at the site of the reoccurring acute pulmonary exacerbations in bronchiectasis. These results reflect the fact that bronchiectasis is a much localised disease and therefore flowover systemic effects are not expected. Focusing on antimicrobial peptides is important due to their antimicrobial capabilities, especially against antibiotic resistant bacteria.

Both vitamin D metabolites, 25-hydroxyvitamin D<sub>3</sub> and 1,25-dihydroxyvitamin D<sub>3</sub>, were shown to be significantly lower in sputum compared to serum. This is most likely due to the majority of vitamin D metabolites being protein bound which limits their distribution into the airways. Lastly, procalcitonin concentrations were not consistently elevated in the serum but were in the sputum of stable bronchiectasis patients. In fact procalcitonin levels were lower than those previously identified in other respiratory conditions. Without knowing the incidences of acute exacerbations, it is unknown whether procalcitonin will be a suitable marker for exacerbations in bronchiectasis.

Sub-optimal vitamin D levels are prevalent in non-cystic fibrosis bronchiectasis patients. Numerous papers report the association between vitamin D deficiency and chronic respiratory diseases. The potential reality is that vitamin D deficiency is a consequence of living with a respiratory condition and not a causative factor. The vitamin D deficiency may be due to decreased outdoor activities and therefore reduced sun exposure as a result of chronic respiratory disease. The only way direct causality can be determined is via the use of randomised controlled trials of vitamin D supplementation. This will reveal if increasing vitamin D status improves the clinical outcome.

The active form, 1,25-dihydroxyvitamin D, has been shown *in vitro* to upregulate both cathelicidin and  $\beta$ -defensin 2. The majority of findings failed to find an association between vitamin D concentrations and antimicrobial peptides *in vivo*. Only CAMP and 1,25-dihydroxyvitamin D localised sputum levels showed a positive correlation. Future attempts at identifying this association *in vivo* should focus on the active hormone 1,25-dihydroxyvitamin D rather than the traditional 25-hydroxyvitamin D.

Other future research should focus on other factors known to influence vitamin D bioavailability, particularly genotyping the genes for vitamin D binding protein and vitamin D receptor in bronchiectasis patients. Further investigation is needed to identify the mechanisms behind the possible synergistic relationships found between cathelicidin/ $\beta$ -defensin 2, and  $\beta$ -defensin 2/procalcitonin. Focus clearly though needs to be on identifying the underlying mechanisms leading to the uneven distribution of antimicrobial peptides. Any future treatment of bronchiectasis needs to be focused on prevention and slowing of the progressive loss of lung function in bronchiectasis patients.

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# Appendices

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## 6.1 Calculation and Statistical Analysis of ELISA Concentrations

ELISA concentrations are determined by curve-fitting the provided standard concentrations. For cathelicidin, hBD-2 and PCT the curve-fit used was a polynomial regression (order=2). For the vitamin D metabolites a four-parameter logistic curve fit is applied. The OD of each duplicate first has the blank OD subtracted, and then is put into the standard curve equation. The dilution factor for the ELISA needs to be taken into account. The dilution factor of three-fold for all sputum samples, resulting from the sputum processing (2.2.2), also needs to be factored into the concentrations. The average concentration can then be found for the duplicates. Duplicates are checked to ensure they are close by calculating the co-efficient of variation (%CV). Only duplicates that meet their respective %CV are included for statistical analysis.

P-values are generated by a simple two-tailed student T-Test for the total samples. A Paired T-test is used when comparing paired sample data between visits. A P-value of  $<0.05$  is considered significant, so rejects the null hypothesis that the means of the two populations are equal. A value  $>0.05$  indicates that the observed result is due to random chance.

Spearman's rank correlation coefficient ( $\rho$ ) enables you to identify if there is a monotonic relationship between two specified variables. The null hypothesis states that there is no association between the two variables. The closer the  $\rho$  value is to  $\pm 1$ , the stronger this relationship.

Data is log transformed to provide a more normal distribution thereby allowing the use of parametric tests. Cathelicidin and hBD-2 concentrations were compared vitamin D concentrations using tertiles as no standardised cut-off levels available.

## 6.1.1 Procalcitonin

PCT concentrations were deduced from their respective standard curves (Figure 39 & Figure 40) using the calculated equations. Neither serum nor sputum samples required dilution prior to PCT ELISA. The blank used for these samples was an empty well. The variation of coefficient used was <40% due to the difficulty in obtaining duplicates samples.

### 6.1.1.1 Standard Curve Run One

The standard curve (Figure 39) was produced using the provided standard (ELISA Kit for Procalcitonin; USCNK, Wuhan, China; Lot: L130118397) providing a concentration range from 31.2-2000pg/mL. The standard curve was fitted using a polynomial trend line (order=2), with the intercept set at zero. The OD value (X-axis) is plotted against the known concentration of the standard (Y-axis). The calculated equation:  $y = 230.23x^2 + 592.5x$ ;  $R^2 = 0.9719$ .

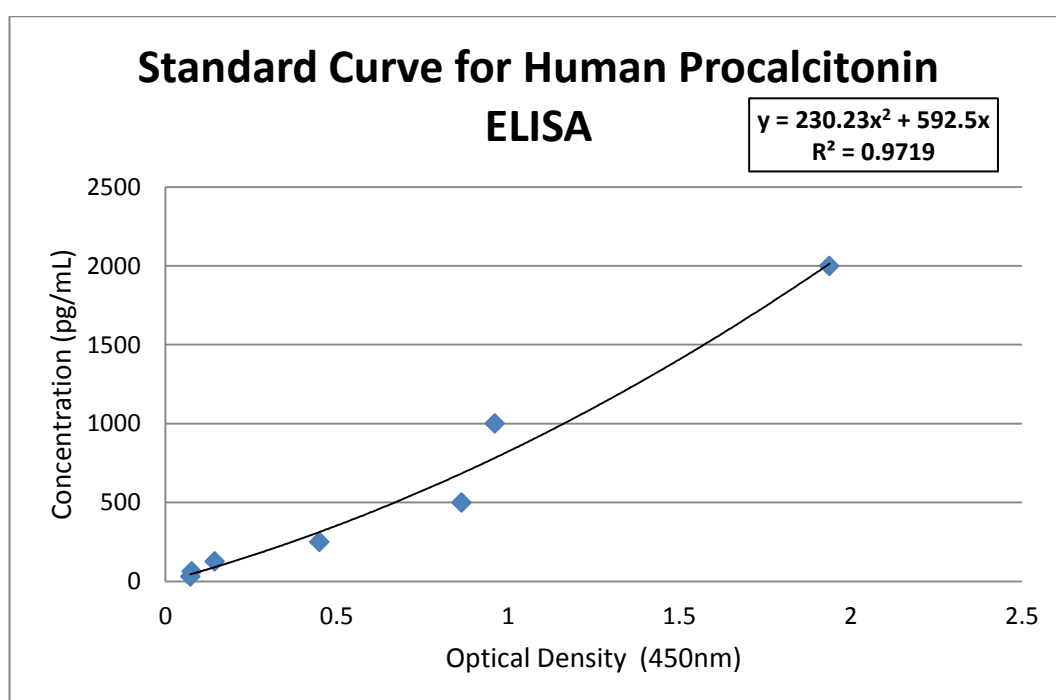


Figure 39 Standard Curve for Human PCT (Run One)

### 6.1.1.2 Standard Curve Run Two

The standard curve (Figure 40) was produced using the provided standard (ELISA Kit for Procalcitonin; USCNK, Wuhan, China; Lot: L130712311), providing a concentration range from 31.2-2000pg/mL. The standard curve was fitted using a polynomial trend line (order=2), with the intercept set at zero. The OD value (X-axis) is plotted against the known concentration of the standard (Y-axis). The calculated equation:  $y = 103.04x^2 + 558.07x$ ;  $R^2 = 0.9997$ .

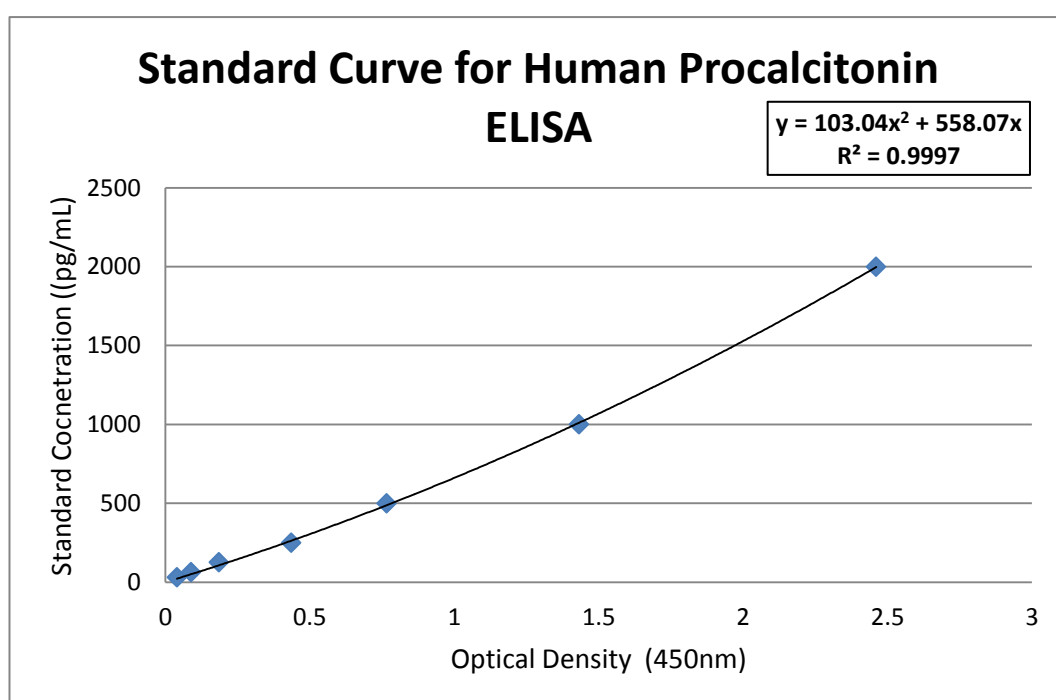
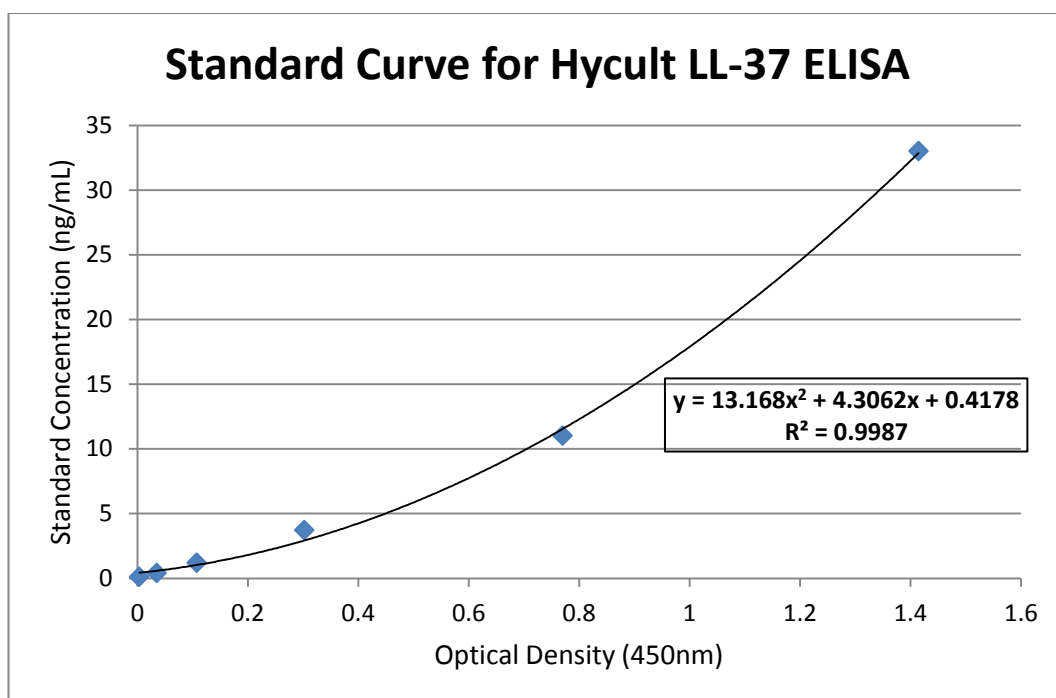


Figure 40 Standard Curve for Human PCT (Run Two)

### 6.1.2 Hycult LL-37 ELISA

The standard for this kit provides a concentration of 0.1-100ng/mL. The following standard curve (Figure 41) was created using a polynomial curve fit (order=2) with intercept not set at zero. The equation:  $y = 13.168x^2 + 4.3062x + 0.4178$  was used to calculate LL-37 concentration, with an  $R^2$  value of 0.9987. The supplied wash/dilution buffer was used for the blank well as used for all standard and sample dilutions. The concentration from the standard curve was multiplied by the serum dilution factor (1:20) before duplicates were averaged, only samples with a %CV of <15% used.



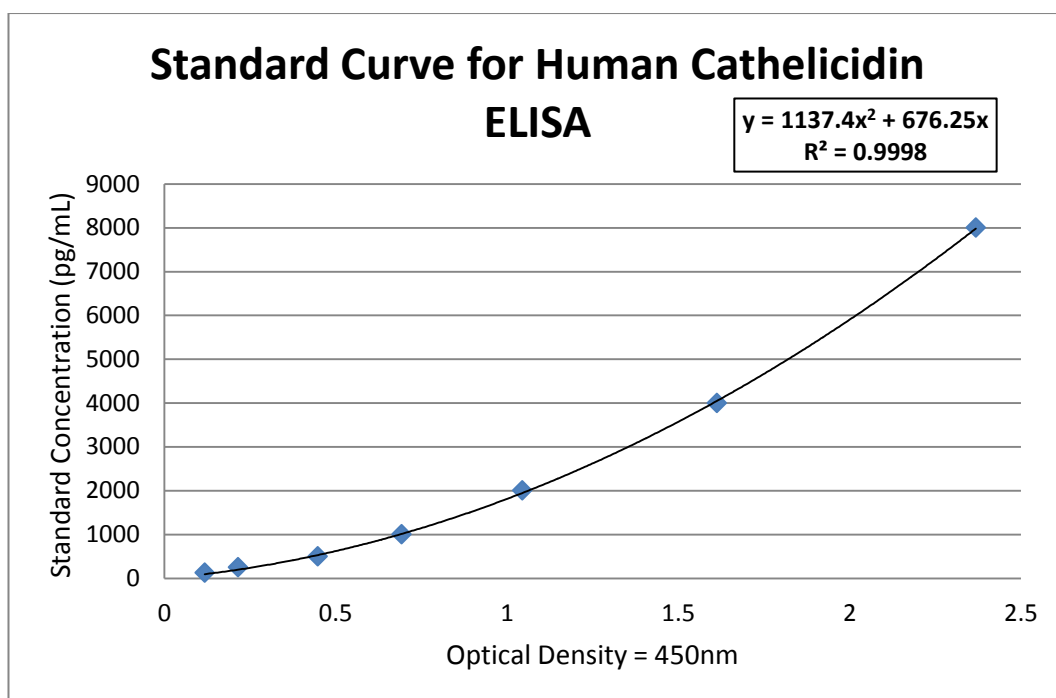
**Figure 41 Standard Curve for Hycult LL-37 ELISA Kit**

### **6.1.3 USCNK CAMP ELISA**

Cathelicidin concentrations were deduced from their respective standard curves (Figure 42 & Figure 43) using the calculated equations. Serum required a 1:500 dilution; sputum samples were diluted 1:5000 for Run One and 1:10000 for Run Two. The blank used for these samples was 0.01mol/L PBS (pH=7.0-7.2) as was used for sample dilutions. Only samples with a % CV < 30% are going to be considered for statistical analysis.

#### **6.1.3.1 Standard Curve Run One**

The standard curve (Figure 42) was produced using the provided standard (ELISA Kit for Cathelicidin Antimicrobial Peptide (CAMP); USCNK, Wuhan, China; Lot: L130205100) providing a concentration range from 125-8000pg/mL. The standard curve was fitted using a polynomial trend line (order=2), with the intercept set at zero. The OD value (X-axis) is plotted against the known concentration of the standard (Y-axis). The calculated equation:  $y = 1137.4x^2 + 676.25x$ ;  $R^2 = 0.9998$ .

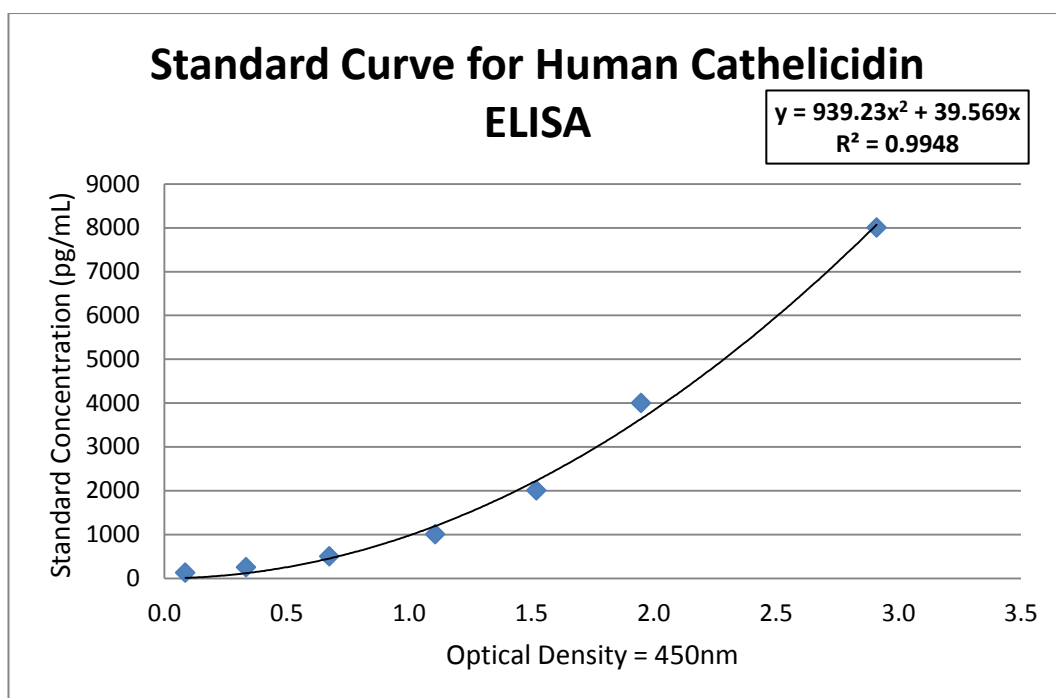


**Figure 42 Standard Curve for Human CAMP ELISA (Run One)**

### 6.1.3.2 Standard Curve Run Two

The standard curve (Figure 43) was produced using the provided standard (ELISA Kit for Cathelicidin Antimicrobial Peptide (CAMP); USCNK, Wuhan, China; Lot: 130712312) providing a concentration range from 125-8000pg/mL. The standard curve was fitted using a polynomial trend line (order=2), with the intercept set at zero. The OD value (X-axis) is plotted against the known concentration of the standard (Y-axis). The calculated equation:  $y = 939.23x^2 + 39.569x$ ;  $R^2 = 0.9948$ .





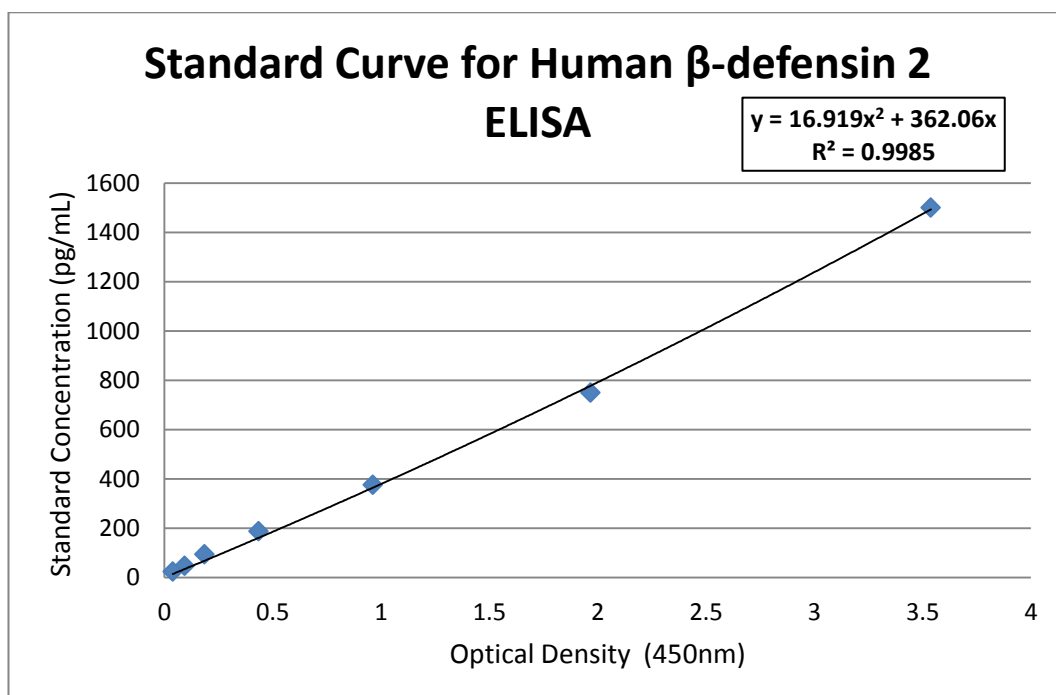
**Figure 43 Standard Curve for Human CAMP ELISA (Run Two)**

#### **6.1.4 $\beta$ -defensin 2**

hBD-2 concentrations were deduced from their respective standard curves (Figure 44 & Figure 45) using the calculated equations. Both sputum and serum samples required no dilution for ELISA assay. The blank used for these samples was an empty well, as no samples had required any further dilutions. Only samples with a % CV < 30% are going to be considered for statistical analysis.

##### **6.1.4.1 Standard Curve Run One**

The standard curve (Figure 44) was produced using the provided standard (ELISA Kit for Defensin Beta 2; USCNK, Wuhan, China; Lot: L130205101) providing a concentration range from 23.44-1500pg/mL. The highest provided standard (3000pg/mL) was omitted from this run, as in preliminary tests it gave an OD reading of 6. In exchange for this standard being omitted a lower standard was added (23.44pg/mL) to the standard curve. The standard curve was fitted using a polynomial trend line (order=2), with the intercept set at zero. The OD value (X-axis) is plotted against the known concentration of the standard (Y-axis). The calculated equation:  $y = 16.919x^2 + 362.06x$ ;  $R^2 = 0.9985$ .

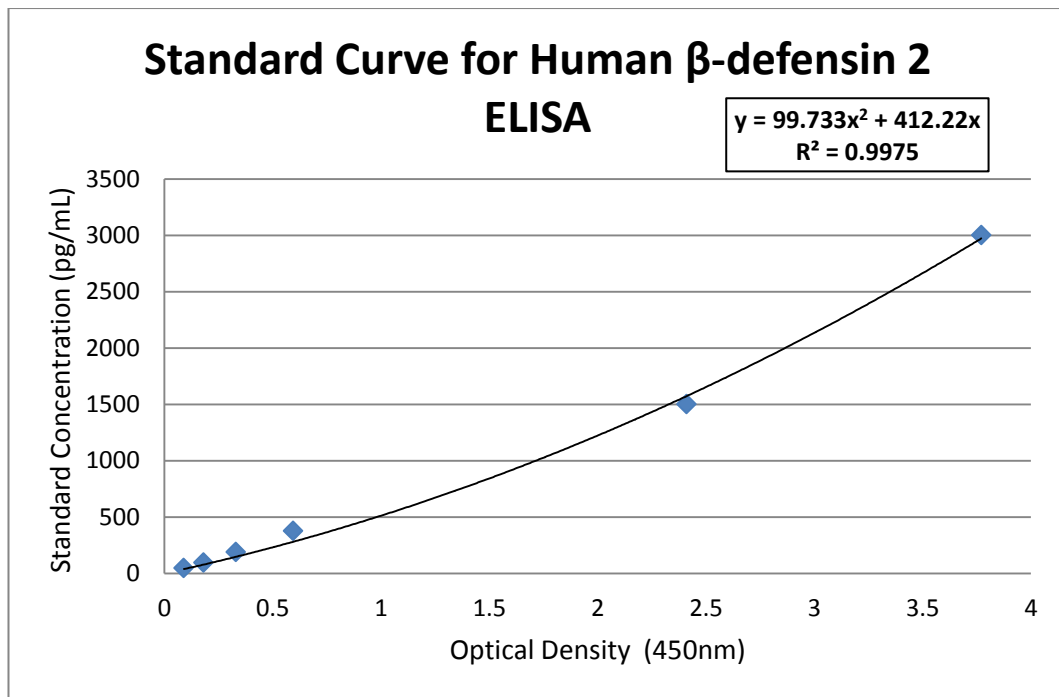


**Figure 44 Standard Curve for hBD-2 (Run One)**

#### **6.1.4.2 Standard Curve Run Two**

The standard curve (Figure 45) was produced using the provided standard (ELISA Kit for Defensin Beta 2; USCNK, Wuhan, China; Lot: L130712312), providing a concentration range from 46.88-3000pg/mL. An outlier in the data, OD for 750pg/mL, was omitted from the standard curve. The standard curve was fitted using a polynomial trend line (order=2), with the intercept set at zero. The OD value (X-axis) is plotted against the known concentration of the standard (Y-axis). The calculated equation:  $y = 99.733x^2 + 412.22x$ ;  $R^2 = 0.9975$ .

Only a quality control was provided with the ELISA kit used for run two (ELISA Kit for Defensin Beta 2; USCNK, Wuhan, China; Lot: L130712312). The quality control measured was 741.88pg/mL. The expected quality control concentration was 540.10pg/mL. The %CV between the observed and expected quality control concentration was 22.26%.



**Figure 45 Standard Curve for hBD-2 (Run Two)**

## 6.1.5 Vitamin D

The 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> concentration has been analysed using a competitive inhibition enzyme immunoassay technique. This type of ELISA requires a non-linear regression for the standard curve to determine samples concentration. The suggested curve-fit was a four-parameter logistic model based on the following equation:

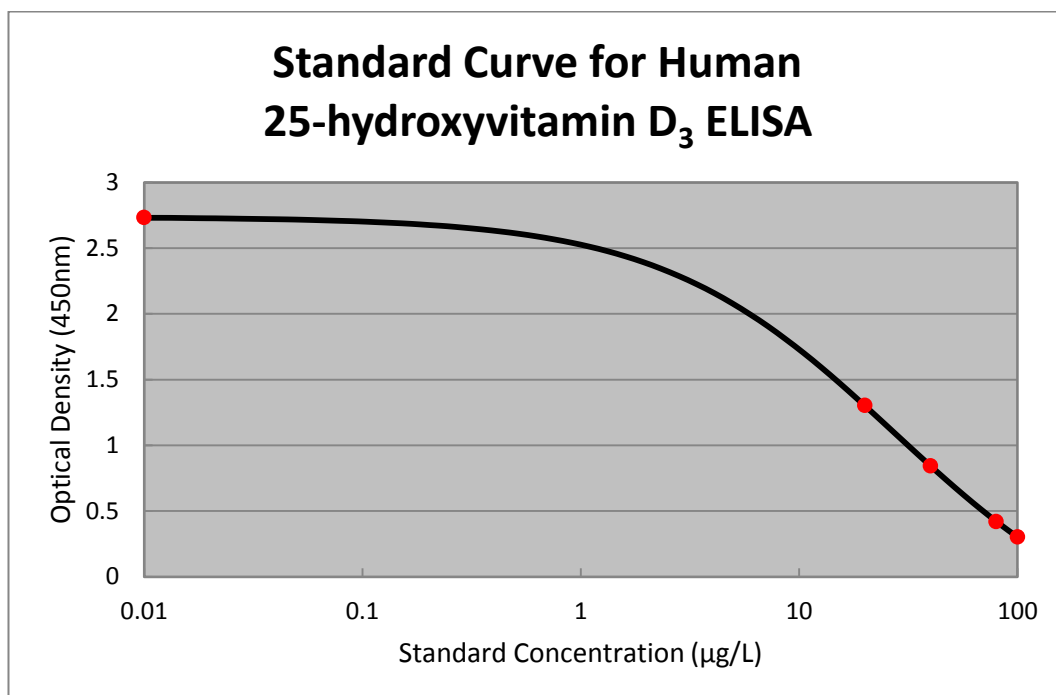
$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b}$$

Concentrations are determined by back-fitting to find x ( $x = (((((B-A)/(y-A)) - 1)^{(1/D))} * C))$ ).

### 6.1.5.1 25-hydroxyvitamin D<sub>3</sub>

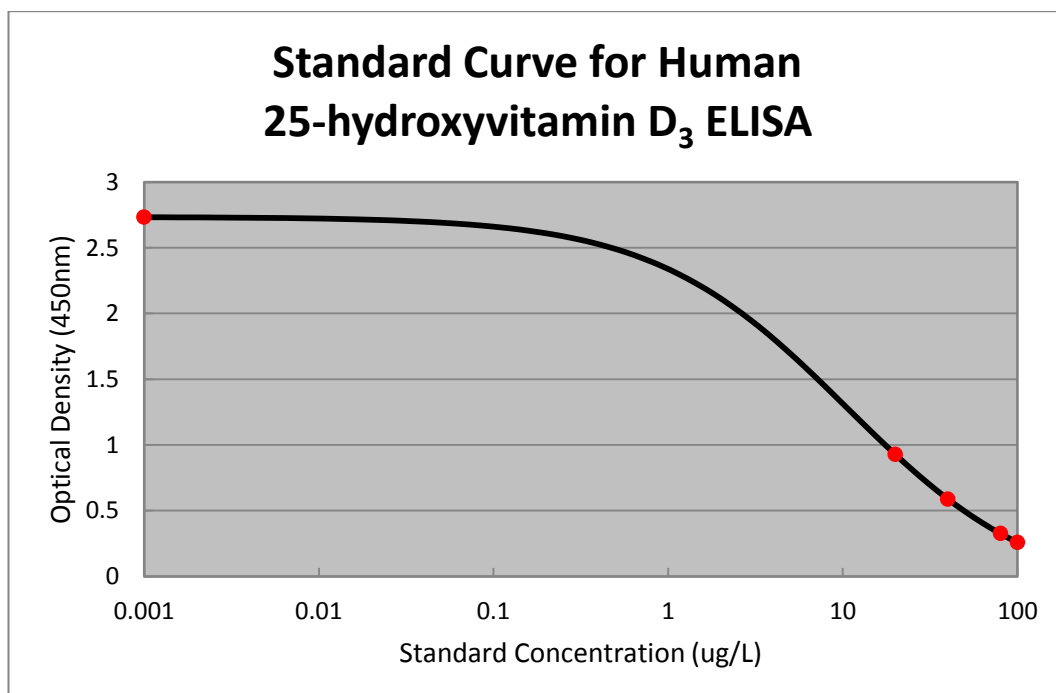
The dilution factor of 1:30 for serum needs to be taken into account and multiplied by the concentration off the standard curve. The supplied standard (Human 25-dihydroxyvitamin D<sub>3</sub> ELISA Kit, CUSABIO, Wuhan, China; Lot: C603130121 & C0724300628) provides a concentration range from 0-100ug/L. The suggested blank used for these samples was a blank well with no solutions

added. Only sample duplicates with a %CV of less than 30% were included for analysis.



**Figure 46 Standard Curve for Human 25(OH)D<sub>3</sub> (Run One)**

The four parameters from Figure 46 are: A=-0.5518, B=-0.8099, C=27.43 and D=2.737 with an R<sup>2</sup> value of 1. The two control values: MVC – 43.07µg/L and HVC - 74.33µg/L.



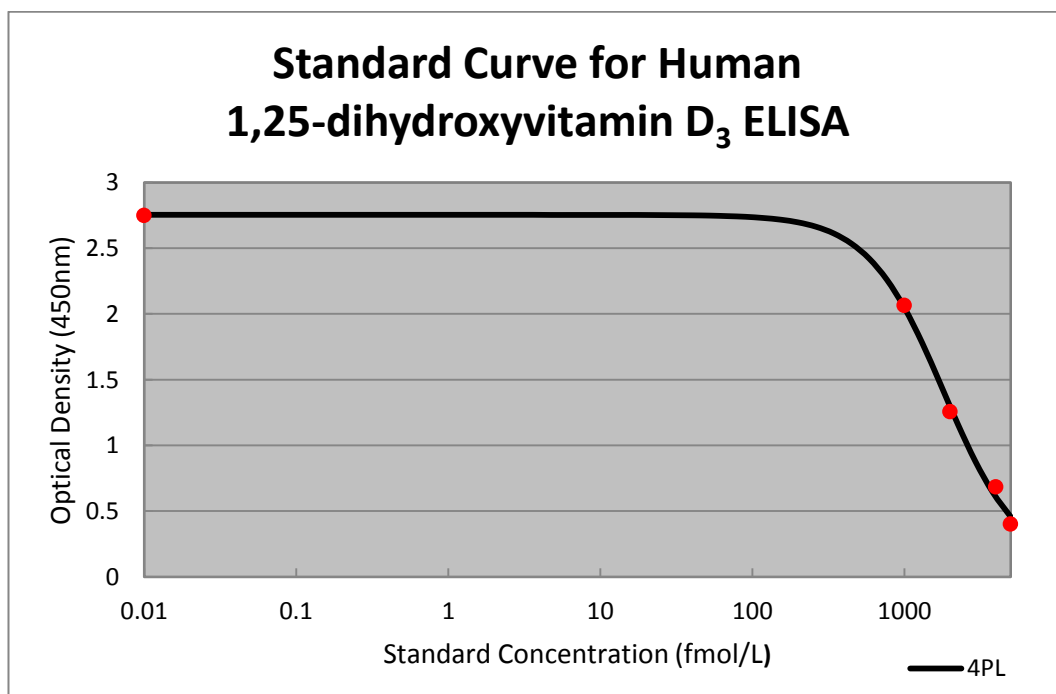
**Figure 47 Standard Curve for Human 25(OH)D<sub>3</sub> (Run Two)**

The four parameters from Figure 47 are: A=-0.1681, B=-0.7829, C=10.55 and D=2.734 with an  $R^2$  value of 1. The two control values: MVC – 45.84 $\mu$ g/L and HVC – 69.49 $\mu$ g/L.

No actual concentrations were provided for the 25(OH)D controls. The %CV of the MVC's was 4.41% for between the two assay runs. The %CV of the HVC's was 4.76% for between the two assay runs.

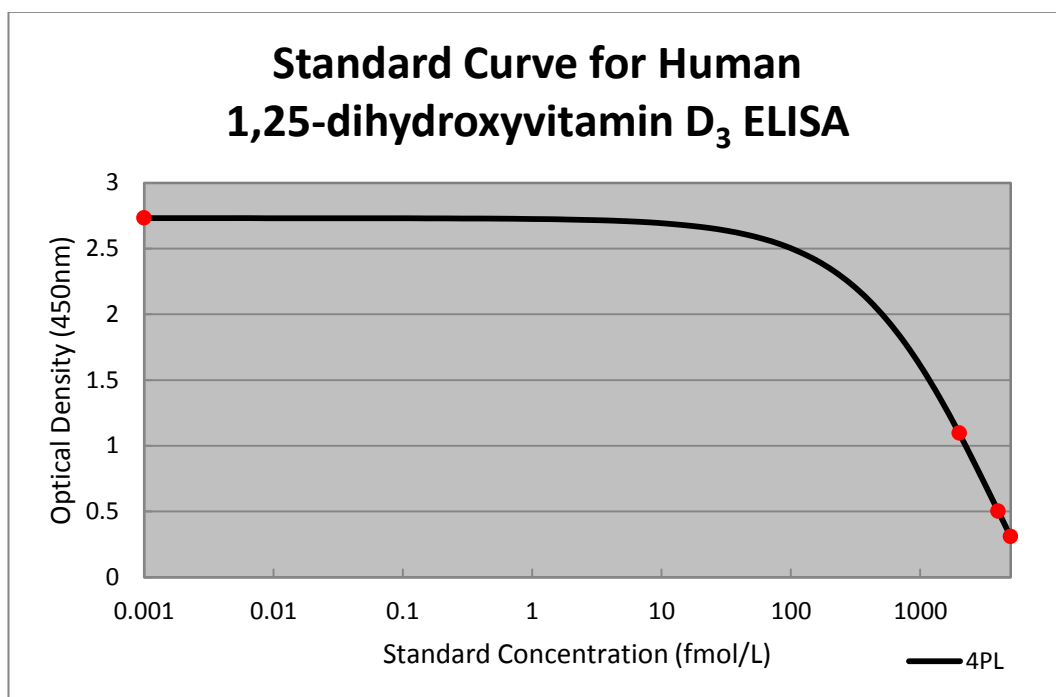
#### 6.1.5.2 1,25-dihydroxyvitamin D<sub>3</sub>

The dilution factor of 1:10 for serum needs to be taken into account and multiplied by the concentration off the standard curve. The supplied standard (Human 1,25-dihydroxyvitamin D<sub>3</sub> ELISA Kit, CUSABIO, Wuhan, China; Lot: C603120903 & C603130422) provides a concentration range from 0-5000fmol/L. The suggested blank used for these samples was a blank well with no solutions added. Only sample duplicates with a %CV of less than 30% were included for analysis.



**Figure 48 Standard Curve for Human 1,25(OH)<sub>2</sub>D<sub>3</sub> (Run One)**

The four parameters from Figure 48 are:  $A=0.0608$ ,  $B=-1.735$ ,  $C=1815$  and  $D=2.754$  with an  $R^2$  value of 0.9971. The two control values: MVC – 2779fmol/L and HVC – 5901fmol/L.



**Figure 49 Standard Curve for Human 1,25(OH)<sub>2</sub>D<sub>3</sub> (Run Two)**

The four parameters from Figure 49 are:  $A=-1.659$ ,  $B=-0.7945$ ,  $C=3849$ ,  $D=2.732$  with an  $R^2$  value of 1. For this standard curve the data point for the 1000fmol/L concentration was excluded to improve the curve fit at the lower optical densities. . The two control values: MVC – 2924fmol/L and HVC – 4960fmol/L.

No actual concentrations were provided for the 1,25(OH)<sub>2</sub>D controls. The %CV of the MVC's was 3.60% for between the two assay runs. The %CV of the HVC's was 12.25% for between the two assay runs.

## 6.2 ELISA Results

**Table 9 Serum ELISA Concentrations**

Visit	25(OH)D <sub>3</sub> (nmol/L)	1,25(OH) <sub>2</sub> D <sub>3</sub> (pmol/L)	hBD-2 (pg/mL)	LL-37 (ng/mL)	CAMP (ng/mL)	PCT (pg/mL)
1	31.95	23.70	107.07	51.98	1440.68	29.25
5	-	26.24	32.85	53.84	-	17.09
6	67.93	46.08	49.47	52.18	-	-
9	32.98	33.15	38.55	40.52	-	-
1	50.82	35.90	137.23	49.96	643.67	-
5	36.93	36.14	48.99	41.19	-	20.56
6	80.33	62.41	73.41	47.50	1632.89	13.27
9	40.16	53.30	79.92	38.56	-	-
1	65.84	36.25	67.69	60.13	543.06	32.52
5	42.47	29.82	46.63	59.35	-	19.63
6	104.06	61.15	36.67	64.26	324.36	9.84
9	61.00	49.26	30.73	56.48	-	-
1	44.52	63.61	25.02	97.29	302.29	13.03
5	-	33.67	26.64	137.49	-	-
6	90.05	70.12	25.43	120.15	227.54	-
9	46.80	51.47	8.05	113.62	-	15.20
1	90.83	55.20	325.00	33.13	692.12	73.34
5	24.59	28.72	217.45	32.01	-	55.27
6	88.66	70.63	172.45	36.10	-	39.33
9	93.94	45.24	153.97	-	-	65.60
1	66.65	52.99	124.46	39.72	409.87	41.11
5	-	34.66	33.17	55.88	401.06	18.96
6	97.04	81.93	79.12	37.18	1315.47	-
9	-	44.69	43.26	-	-	25.69
1	27.42	24.49	101.08	86.45	542.33	83.10
5	28.21	13.17	173.56	65.19	1760.61	153.92

6	19.21	21.43	93.94	-	-	-
9	35.01	19.92	93.89	-	-	22.32
1	108.33	69.07	91.06	61.30	295.63	27.10
5	49.08	36.73	248.73	70.10	710.40	68.67
6	97.26	94.57	94.13	-	-	42.04
9	129.59	40.21	183.10	-	-	16.44
1	75.69	34.03	18.75	50.91	415.47	24.66
5	65.93	32.82	217.72	45.73	-	733.08
6	97.18	76.94	35.72	-	-	-
9	77.73	31.55	33.77	-	-	15.54
1	74.49	81.75	21.66	33.36	669.63	141.79
5	80.05	62.08	66.24	25.91	860.73	10.02
6	93.38	64.95	44.46	-	164.98	-
9	60.75	27.52	22.18	-	-	14.78
1	63.74	-	-	-	-	142.75
5	81.37	44.96	65.19	-	693.52	-
6	79.49	46.90	-	-	-	-
1	48.40	61.46	116.57	23.35	264.65	70.66
5	-	-	154.28	23.32	285.26	-
1	68.11	63.97	219.82	24.82	156.33	179.79
5	78.19	46.17	233.21	27.53	-	-
6	71.31	53.50	51.08	-	89.41	9.26
9	56.73	28.84	45.04	-	-	-
1	102.81	67.86	-	127.36	245.02	103.18
5	-	-	109.36	107.42	290.44	-
5	-	-	72.35	-	341.49	-
1	83.56	65.70	-	-	653.45	47.71
5	-	-	52.24	-	816.49	-
1	91.24	59.29	144.67	-	256.82	-
5	-	-	-	-	-	12.09
1	76.55	76.76	72.04	-	-	71.91
5	-	-	59.41	-	-	-



5	-	-	-	-	-	8.22
1	-	54.59	110.82	-	-	-
5	-	53.93	230.08	-	590.95	26.39
1	105.61	-	266.42	48.37	175.32	35.09
5	83.21	-	-	46.79	-	28.06
6	69.03	74.45	-	-	-	-

**Table 10 Sputum ELISA Concentrations**

<b>Visit</b>	<b>25(OH)D<sub>3</sub> (nmol/L)</b>	<b>1,25(OH)<sub>2</sub>D<sub>3</sub> (pmol/L)</b>	<b>hBD-2 (pg/mL)</b>	<b>CAMP (µg/mL)</b>	<b>PCT (pg/mL)</b>
1	19.12	19.07	201.47	-	-
5	24.11	18.10	93.35	-	128.68
6	23.55	18.18	328.19	196.55	252.23
9	19.24	17.46	376.69	-	338.59
1	13.63	12.22	107.99	40.68	510.55
5	16.14	14.24	1090.66	-	697.48
6	9.44	12.66	2121.03	-	653.03
9	11.70	11.76	1493.29	93.09	690.67
1	13.43	11.58	38.68	62.40	60.64
5	24.91	18.10	46.04	-	205.71
6	24.11	16.17	275.08	-	282.21
9	14.41	14.49	137.45	133.75	278.19
1	6.00	14.03	96.64	-	176.91
5	11.43	31.98	178.01	-	151.68
6	21.79	17.60	149.83	-	393.26
9	31.88	17.41	73.56	-	132.44
1	9.38	14.36	68.69	64.06	131.75
5	19.56	39.18	103.43	115.24	139.45
6	27.52	15.25	157.82	63.83	211.39
9	21.39	13.49	574.20	-	753.04
1	18.23	15.16	65.58	35.61	78.66
5	19.95	13.15	262.28	18.18	228.71

6	16.09	8.38	376.02	142.34	280.49
9	16.92	12.25	262.96	-	-
1	6.68	7.38	74.65	-	-
5	-	-	89.57	44.92	170.08
6	25.94	10.39	136.23	58.09	178.77
9	13.91	11.08	170.85	-	166.61
1	11.12	-	43.86	55.73	-
5	11.20	9.98	72.24	76.09	167.49
6	12.11	10.84	149.77	-	145.20
9	15.63	9.45	749.44	-	530.47
1	11.60	5.81	90.50	-	248.26
5	4.83	8.99	150.11	46.31	223.03
6	4.91	10.07	211.11	-	297.82
9	7.82	7.25	405.46	41.66	-
1	19.82	8.37	146.25	23.97	-
5	9.91	7.79	81.84	-	176.33
6	-	8.09	56.95	-	-
9	9.03	7.02	262.69	21.37	252.02
1	13.40	15.77	-	76.00	-
1	14.40	21.14	57.66	145.18	499.80
5	-	9.19	-	-	-
1	7.61	14.78	94.88	5.64	247.54
5	9.25	8.45	276.77	-	394.91
6	8.54	8.18	100.66	5.70	229.46
9	6.93	6.39	192.91	-	194.02
1	17.15	16.60	79.84	-	246.30
1	28.25	42.96	-	61.33	164.21
1	20.42	19.94	50.40	-	114.26
1	-	4.23	-	2.17	133.92
1	22.00	-	-	-	81.57
1	-	-	-	-	152.22
5	-	-	36.23	-	-

1	0.58	0.82	152.54	-	570.89
1	17.83	-	-	-	104.33
1	-	-	30.07	-	195.43
1	-	-	129.76	1.48	360.72
1	12.36	-	56.57	0.61	119.02